Flavobacterium psychrophilum - Experimental challenge and immune response

Henriksen, Maya Maria Mihályi

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Flavobacterium psychrophilum

Experimental challenge and immune response

Maya Maria Mihályi Henriksen

PhD Thesis

2013

National Veterinary Institute
Section for Bacteriology, Pathology and Parasitology
Technical University of Denmark, Copenhagen

The thesis is submitted for the degree of Doctor of Philosophy and is based on a review of the existing literature and 3 scientific papers.

DTU Vet
National Veterinary Institute

Supervisors:
Associate professor Inger Dalsgaard
Technical University of Denmark, Frederiksberg, Denmark
Professor Kurt Buchmann
University of Copenhagen, Frederiksberg, Denmark
Associate professor Per Walter Kania
University of Copenhagen, Frederiksberg, Denmark
Professor Niels Lorenzen
Aarhus University, Aarhus, Denmark

Assessment committee:
Professor Niels Jørgen Olesen (chairperson)
Technical University of Denmark, Frederiksberg, Denmark
Head of Laboratory Tom Wiklund
Åbo Akademi University, Åbo, Finland
Professor Emeritus Bent Aasted
University of Copenhagen, Frederiksberg, Denmark

Front cover artwork:
Flavobacterium psychrophilum by Maya Henriksen

Back cover artwork:
Rainbow trout fry by Maya Henriksen

***Flavobacterium psychrophilum* – challenge and immune response**

Printed by Rosendahls – Schultz Grafisk

Table of contents

Table of contents 1
Preface 2
Summary (English) 3
Sammendrag (dansk) 5
List of abbreviations 7
Introduction 8
1. *Flavobacterium psychrophilum* 10
1.1. History and taxonomy 10
1.2. Morphology and biochemical characteristics 11
1.3. Cultivation and serology 12
1. 4. The disease RTFS / BCWD 14
2. Experimental infections 19
3. The fish immune system 29
3.1. Lymphoid organs 30
3.2. Principles of innate immunity 33
3.3. Inflammation 40
3.4. Adaptive immune response 41
4. Immune response in rainbow trout to *F. psychrophilum* 45
5. Aquaculture 51
5.1. Rainbow trout 51
5.2. Farming, health and management 51
5.3. Hydrogen peroxide and the gills 53
5.4. Stress 55
6. Methodological considerations 59
7. Concluding remarks and perspectives for future research 63
Acknowledgements 64
References 65
Presentations 75
Accompanying papers 76

Preface

The research presented in this PhD has been conducted primarily in Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, in collaboration with Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen from July 2010 to October 2013. This work was performed under the Danish Fish Immunology Research Centre and Network (DAFINET) and was supported by the Danish Council of Strategic Research and the National Veterinary Institute, Technical University of Denmark, Copenhagen.

The thesis is organized as a literature review of immunology, *Flavobacterium psychrophilum*, rainbow trout fry syndrome and interrelated topics combined with articles, which have been published or are submitted for publication in peer reviewed international journals during the PhD.

The present thesis is based on the following original publications, which are referred to by Roman numerals I-III:

I.
Henriksen MMM, Madsen L, Dalsgaard I (2013). ***Effect of Hydrogen Peroxide on Immersion Challenge of Rainbow Trout Fry with Flavobacterium psychrophilum***. PLoS ONE 8(4): e62590. doi:10.1371/journal.pone.0062590

II.
Henriksen MMM, Kania PW, Dalsgaard I, Buchmann K (2013). ***Evaluation of the immune response in rainbow trout fry, Oncorhynchus mykiss* (Walbaum)*, after waterborne exposure to Flavobacterium psychrophilum and/or hydrogen peroxide***. Journal of Fish Diseases. Accepted.

III.
Henriksen MMM, Kania PW, Dalsgaard I, Buchmann K (2013). ***Effect of hydrogen peroxide and/or Flavobacterium psychrophilum on the gills of rainbow trout fry, Oncorhynchus mykiss* (Walbaum)**. Submitted manuscript.

Summary (English)

The disease rainbow trout fry syndrome (RTFS) is caused by the bacterial fish pathogen *Flavobacterium psychrophilum*. It has been the cause of great losses of rainbow trout in aquacultures both in Denmark and around the world. It was estimated that RTFS resulted in the death of 88 million fry in 1998, which amounted to a loss of 18 million DKK. Disease outbreaks are typically seen at water temperatures below 15 °C and typically with fry mortality rates from 50-60 %.

Disease outbreaks are usually first observed shortly after the onset of feeding after the yolk sack is depleted. At this point the immune system of the fry is not fully developed. Theoretically, the infection pressure could be subdued by vaccinating larger fish, but no commercial vaccine is yet available. Diagnostic methods are well described and the disease is treated with antibiotics. To prevent disease outbreaks and subsequent use of antibiotics, further knowledge of the disease is needed.

Previous studies focusing on various types of aquacultures demonstrated the presence of *F. psychrophilum* in all examined farms. The bacterium was demonstrated in gills, skin, internal organs and wounds both during RTFS outbreaks and periods without disease.

The main purpose and focus of the present thesis was to increase knowledge of the immune response following infection with *F. psychrophilum*, which may contribute to the future development of vaccines and other preventive measures. The project consisted of three main tasks: 1) Establish an experimental infection model imitating natural infection, 2) examine the immune response in blood and selected organs, and 3) examine potential portals of entry for the bacterium.

Previous experimental immersion-challenges involving *F. psychrophilum* have resulted in none or low mortality in rainbow trout fry, unless the fish are stressed or have their surface compromised through e.g. injuries to the skin. The effect of a range of hydrogen peroxide (H_2O_2) concentrations was tested on fry in order to assess mortality. An appropriate dose was subsequently combined with immersion in a diluted bacterial broth. The method was shown to increase the mortality of RTFS significantly and as a result, this model for infection was used for the planned studies. All subsequent investigations thus include two infected groups, one of which was pretreated with hydrogen peroxide, while the other was not.

Real-time PCR (RT-PCR) was used to examine the immune response in the head kidney during the first eight days after infection, and enzyme-linked immunosorbent assay (ELISA) was used to evaluate the production of antibodies 50 days post-exposure. A pro-inflammatory response was observed in both groups infected with *F. psychrophilum*. However, only a weak pattern was observed in the regulation of the adaptive response, while numerous correlations between the regulation of genes and the amount of *F. psychrophilum* 16S rRNA were demonstrated. Exposure to H_2O_2 before immersion in *F. psychrophilum* also influenced correlation between regulation of genes and pathogen

load in several cases. Pre-treatment with H_2O_2 e.g. delayed the positive correlation between IgM and pathogen load in the head kidney. Antibodies against *F. psychrophilum* were present in the blood of both infected groups 50 days after infection, but no significant difference could be observed at this time point.

Morphological changes in the gill tissue after exposure to *F. psychrophilum* and H_2O_2 was examined on tissue sections stained with hematoxylin and eosin (H&E). Exposure to *F. psychrophilum* or H_2O_2 resulted in epithelial lifting and formation of edemas, but in both cases the tissue was regenerating after 192 hours. However, when the fish had been exposed to both H_2O_2 and *F. psychrophilum*, the damage was still evident at this time point. The relative pathogen load measured as 16S rRNA was highest at the first sampling and decreased steadily with no significant effect from pre-treatment with H_2O_2. No bacteria were observed on the H&E-stained tissue or visualized using a *F. psychrophilum*-specific probe with fluorescence in situ hybridization. Changes in gene expression in the gills after exposure to *F. psychrophilum* and H_2O_2 was also examined using RT-PCR. The observed immune response was limited, and neither a typical T_h1 or T_h2 response was observed.

The results indicate that immersion in *F. psychrophilum* may suppress the immune response in its host and induce lesions in the gills. A single exposure to H_2O_2 influenced the immune response to subsequent exposure to *F. psychrophilum* in both head kidney and gills, and leads to increased mortality. The results show a need for further investigation regarding the potential connection between routine non-medical treatments like H_2O_2 in aquaculture and disease outbreaks.

Sammendrag (dansk)

Bakterien *Flavobacterium psychrophilum* er årsag til sygdommen ”yngeldødelighedssyndromet” (YDS), som siden midten af 80erne har medført store tab af opdrættede regnbueørreder i både Danmark og resten af verden. I 1998 vurderedes det, at tabene på grund af YDS alene kunne opgøres til 88 millioner yngel døde i 1998, hvilket medførte et økonomisk tab på 18 millioner DKK. Udbrud af sygdommen ses typisk ved vandtemperaturer under 15 °C og med en typisk yngeldødelighed på 50-60 %.

Sygdomsudbrud ses typisk første gang kort tid efter opstart af fodring efter næringen i blommesækken er opbrugt. På dette tidspunkt er regnbueørredens immunsystem endnu ikke fuldt udviklet. Teoretisk set kan større fisk vaccineres for at holde smittetrykket nede, men endnu eksisterer der ingen kommercielle vacciner imod YDS. Diagnostiske metoder til påvisning af sygdommen er velkendte, og YDS behandles med antibiotika. For at begrænse fremtidige sygdomsudbrud og den medfølgende anvendelse af antibiotika er det nødvendigt at opnå større viden om YDS.

Tidligere projekter med udgangspunkt i forskellige typer af dambrug har vist, at *F. psychrophilum* fandtes på samtlige af de undersøgte dambrug. Både i forbindelse med YDS-udbrud og i perioder uden sygdom kunne bakterien påvises på gæller, hud, indre organer og sår.

Hovedformålet med denne afhandling var at øge viden omkring immunforsvaret efter infektion med *F. psychrophilum*, hvilket kan bidrage til den fremtidige udvikling af vacciner og andre forebyggende tiltag. Projektet bestod af tre hovedopgaver: 1) At etablere en eksperimentel infektionsmodel, der imiterer naturlig smitte, 2) at undersøge immunresponsen i blod og udvalgte organer samt 3) at undersøge mulige indgangspunkter for bakterien.

Tidligere laboratorieforsøg med eksperimentel *F. psychrophilum* badinfektion har resulteret i ingen eller lav dødelighed i regnbueørred, med mindre fiskene stresses eller får kompromitteret deres overflade ved f.eks. at påføre huden skader. Effekten af en række koncentrationer af brintoverilte (H_2O_2) blev testet på fiskeyngel for at undersøge eventuel dødelighed. En passende dosis af H_2O_2 blev efterfølgende kombineret med bad i en fortyndet bakteriebouillon. Denne fremgangsmåde viste sig at hæve dødeligheden af YDS signifikant, hvorefter modellen blev brugt til de planlagte undersøgelser. I alle efterfølgende forsøg indgår der således to smittede grupper; en med og en uden forbehandling med H_2O_2.

Real-time PCR (RT-PCR) blev anvendt til at belyse immunresponsen i hovednyren i de første otte dage efter smitte, og enzyme-linked immunosorbent assay (ELISA) blev brugt til at vurdere dannelsen af antistof 50 dage efter eksponering. Overordnet set blev en pro-inflammatorisk respons observeret i begge *F. psychrophilum*-inficerede grupper. Dog blev der kun set et svagt

sammenhængende mønster i reguleringen af den adaptive respons, mens flere korrelationer imellem reguleringen af gener og mængden af *F. psychrophilum* 16S rRNA blev påvist. I flere tilfælde påvirkede eksponering for H_2O_2 ligeledes korrelationen mellem regulering af gener og bakteriemængde. Forbehandling med H_2O_2 medførte f.eks. en forsinkelse af den positive korrelation mellem patogenmængde og IgM. Dog var der ingen forskel i mængden af antistof i blodet efter 50 dage.

Morfologiske ændringer i gællevæv efter eksponering for *F. psychrophilum* og H_2O_2 blev undersøgt på vævsprøver farvet med hematoxylin og eosin (H&E). Eksponering for enten *F. psychrophilum* eller H_2O_2 resulterede i løft af epitelet og ødemdannelse, men i begge tilfælde var vævet ved at blive gendannet efter 192 timer. Derimod var væv taget fra fisk eksponeret for både H_2O_2 og *F. psychrophilum* stadig skadet på dette tidspunkt. Den relative bakteriemængde målt som 16S rRNA var på sit højeste ved første prøveudtagning og faldt herefter støt uden signifikant effekt af forbehandling med H_2O_2. Der observeredes hverken bakterier på de H&E-farvede vævsprøver eller ved brug af en *F. psychrophilum*-specifik probe til fluorescence in situ hybridization. Ændringer i genekspression i gællerne efter eksponering for *F. psychrophilum* og H_2O_2 blev også undersøgt ved hjælp af RT-PCR. Den observerede immunreaktionen var begrænset, og hverken et typisk T_h1 eller T_h2 respons blev observeret.

Resultaterne indikerer at *F. psychrophilum* kan hæmme fiskens immunreaktion og forårsage læsioner på gællerne. En enkelt eksponering for H_2O_2 påvirkede immunreaktionen på efterfølgende eksponering for *F. psychrophilum* i hovednyren såvel som i gællerne og medførte en højere dødelighed. Dette belyser behovet for yderligere forskning vedrørende den potentielle sammenhæng mellem sygdomsudbrud og rutinemæssig brug af hjælpestoffer i dambrug.

List of abbreviations

BCWD	Bacterial cold water disease
BGD	Bacterial gill disease
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CFU	Colony forming units
CRP	C-reactive protein
CTL	Cytotoxic T-lymphocyte
dsRNA	Double stranded RNA
ELF-1α	Elongation factor 1-α
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescent *in situ* hybridization
FAO	Food and Agriculture Organisation
GPI	Glycosylphosphatidylinositol
H_2O_2	Hydrogen peroxide
H&E stain	Hematoxylin and eosin stain
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeats
MBL	Mannose-binding lectin
MHC	Major histocompatibility locus
mRNA	Messenger ribonucleic acid
NGD	Nodular gill disease
OMV	Outer membrane vesicle
OD	Optical density
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PolyI:C	Polyinosinic:polycytidylic acid
RAG	Recombination activating gene
RNA	Ribonucleic acid
RTFS	Rainbow trout fry syndrome
RT-PCR	Reverse transcriptase quantitative polymerase chain reaction
SAA	Serum amyloid A
SAP	Serum amyloid P
ssRNA	Single stranded RNA
T_CR	T-cell receptor
T_{reg}	Regulatory T-cells
TYES	Tryptone yeast extract salts
VLR	Variable lymphocyte receptors

Introduction

The Food and Agriculture Organisation (FAO) of the United Nations estimates that approximately one billion people rely on fish as their primary source of animal protein, especially in regions with little production of livestock. The demand is increasing, primarily in Asia, Africa and South America. Aquaculture production has therefore increased rapidly and is now the world's fastest growing food-producing industry (Tidwell & Allan 2001; FAO 2000).
Disease outbreaks are a major problem in aquacultures, leading to losses in production worldwide. Due to the development of vaccines, the use of antibiotics has already declined (Tidwell & Allan 2001). However, excessive use of antibiotics in aquaculture is still a problem, especially in developing countries, and has resulted in the emergence of antibiotic-resistant bacteria (Cabello 2006). Consumers are becoming increasingly aware of the impact of antibiotic resistance on human health and food safety. Thus efforts are needed to move away from the use of antibiotics by developing new vaccines and considering alternative strategies for combating disease, such as bacteriophage therapy and pre-[1] and probiotic[2] products.

The bacterial fish pathogen *Flavobacterium psychrophilum* is the causative agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD). The pathogen is a major cause of disease in aquacultures and has led to great losses of especially rainbow trout, both in Denmark and worldwide (Dalsgaard & Madsen 2000; Nematollahi, Decostere, Pasmans & Haesebrouck 2003). *F. psychrophilum* affects most salmonid fish, and rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*) and ayu (*Plecoglossus altivelis*) are the most susceptible species. Other species, such as eel (*Anguilla anguilla*), can also be affected by the disease (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). Disease outbreaks are typically seen at water temperatures below 15 °C. Fry are most susceptible and mortality rates can range up to 90 % (Lorenzen 1994).

A commercial vaccine is to our knowledge not yet available and the disease is currently treated with antibiotics. Vaccination may contribute to prevent RTFS, but more knowledge concerning the immune response to the pathogen is needed to achieve this goal. Despite the serious impact of *F. psychrophilum* on rainbow trout farming, the route of infection and host immune response are still largely unknown. This can be partly attributed to challenges in laboratory work with *F. psychrophilum*, especially issues concerning the establishing of a reproducible immersion-based model for infection. Although experimental infection using injection has been standardized (Madsen & Dalsgaard 1999), it has been difficult to attain reproducible mortality using an experimental immersion-based model (Decostere, Lammens & Haesebrouck 2000). Consequently, all previous investigations concerning the immune response have relied on either naturally infected or injection-challenged fish. The mucosal surfaces are bypassed with injections, and many external factors may potentially confound interpretation of results obtained using naturally infected animals.

[1] Prebiotic: Feed ingredient, which is not digested but stimulates benign bacteria in the digestive system.
[2] Probiotic: Live bacteria, which are beneficial for health, such as lactic acid bacteria in human food.

In aquaculture, various non-medical pharmaceutical products are used against parasites like white spot disease (*Ichthyophthirius multifiliis*) and the freshwater mould *Saprolegnia*. Formalin has previously been used, but is to be phased out of use in Denmark due to human health considerations. Hydrogen peroxide (H_2O_2) has been investigated as a potential replacement (Jokumsen & Svendsen 2010). Although H_2O_2 is generally considered to be mostly harmless, treatment at recommended concentrations levels results in damages on gills and changes in regulation of immune-relevant genes (Tort, Jennings-Bashore, Wilson, Wooster & Bowser 2002; MMAH II; MMAH III).

The aim of the project was to examine the immune response associated with *F. psychrophilum* infection in rainbow trout fry, to provide the requisite knowledge for improving vaccination and/or immune stimulation. Because *F. psychrophilum* is primarily a problem in fry, rainbow trout weighing approx. 1 g were used for all investigations in the present study. Activation of both adaptive immunity and development of protective immunity have previously been observed at early life stages (Chettri, Raida, Kania & Buchmann 2012; Buchmann, Nielsen & Nielsen 2003). An immersion-based experimental challenge model was established (MMAH I), and exposure to H_2O_2 before immersion in *F. psychrophilum* was used to increase mortality of subsequent *F. psychrophilum* infection. Thus, information regarding the effect of H_2O_2 as a treatment in aquaculture was also obtained. The pathogenesis of *F. psychrophilum* in rainbow trout is poorly understood, and changes in expression of immune relevant genes in the gills and head kidney of experimentally infected rainbow trout fry were examined with qPCR. Using enzyme-linked immunosorbent assay (ELISA), the level of *F. psychrophilum*-specific antibodies in plasma 50 days after infection was determined (MMAH II). The gills were examined as a potential portal of entry for *F. psychrophilum*. Tissue sections of gills and heads were stained with hematoxylin and eosin (H&E) to examine morphological changes in the tissue (MMAH III). Fluorescence in situ hybridization (FISH) was also carried out on the tissue sections to localize the bacteria.

1. *Flavobacterium psychrophilum*

1.1. History and taxonomy

Flavobacterium psychrophilum is a Gram negative fish pathogen of the family *Flavobacteriaceae* within the Bacteroidetes phylum (Table 1). The rod-shaped bacterium was originally isolated from the kidney and external lesions on a diseased juvenile coho salmon in West Virginia, USA. The bacteria was initially named *Cytophaga psychrophila*, based on its biochemical properties (Borg 1960), but was later reclassified based on DNA homology and renamed *Flexibacter psychrophilus* (Bernardet & Grimont 1989). Based on the results of G+C content, fatty acid and protein analysis in addition to DNA-rRNA hybridization, it was finally transferred to the family *Flavobacteriaceae* and named *F. psychrophilum* (Bernardet *et al.* 1996).

Domain	Bacteria
Phylum	Bacteroidetes
Class	Flavobacteria
Order	Flavobacteriales
Family	*Flavobacteriaceae*
Genus	*Flavobacterium*

Table 1. Classification of *F. psychrophilum*.

The disease resulting from infection with *F. psychrophilum* was called bacterial cold water disease (BCWD), because outbreaks were most common at water temperature below 10 °C (Borg 1960), where it caused significant mortalities in salmonids; especially coho salmon and rainbow trout (Holt, Rohovec & Fryer 1993). In the 1980s, *F. psychrophilum* caused systemic disease in juvenile rainbow trout in Europe and was called rainbow trout fry syndrome (RTFS) (Lorenzen *et al.* 1991; Madsen & Dalsgaard 1998). Both terms are still used. Originally, the distribution of *F. psychrophilum* was thought to be limited to North America, but it has since been found in freshwater aquacultures all around the world (Nematollahi, Decostere, Pasmans & Haesebrouck 2003), and RTFS still has a significant impact on rainbow trout and other salmonid fish in aquacultures worldwide (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). Besides horizontal transfer between fish, *F. psychrophilum* may be vertically transmitted from brood fish to eggs (Madsen, Møller & Dalsgaard 2005), which suggests that the global spread of RTFS could be due to transmission from trade with live fish and eggs.

All salmonid fish are expected to be susceptible to infection with *F. psychrophilum*. Infection has been reported in many species: Sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*), masou salmon (*Oncorhynchus*

masou), lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*) and cutthroat trout (*Oncorhynchus clarki*). However, rainbow trout and coho salmon seem the most susceptible species. *F. psychrophilum* can also cause disease in non-salmonids, such as ayu, eel (*Anguilla Anguilla*) and cyprinids. (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). Furthermore, the bacterium has been isolated from fish without clinical signs of disease (Madsen *et al.* 2005; Madetoja, Dalsgaard & Wiklund 2002). These findings suggest that a variety of species may act as reservoirs of infection for more susceptible salmonid species.

1.2. Morphology and biochemical characteristics

F. psychrophilum cells are flexible and slender rods with round ends, and actively growing cells measure approximately 0.3-0.75 x 1.5-7.5 µm (Nematollahi, Decostere, Pasmans & Haesebrouck 2003; Holt *et al.* 1993; Dalsgaard 1993). Both size and morphology are age-dependent; younger cells are generally longer, while older cells are occasionally observed to have swollen segments or to curl up in 'ring'-like formations (Vatsos, Thompson & Adams 2003). Slow gliding motility has been reported in some strains[3] (Nematollahi, Decostere, Pasmans & Haesebrouck 2003).

Flavobacterium psychrophilum can secrete different proteolytic enzymes. These can degrade collagen, fibrinogen, casein, gelatin, elastin, chondroitin sulphate, tributyrin, tyrosin, haemoglobin and fish muscle extract, while carbohydrates cannot be utilized (Dalsgaard & Madsen 2000; Nematollahi, Decostere, Pasmans & Haesebrouck 2003). The primary role of these proteases may be in pathogenesis through damage or alteration of the host tissue or acquisition of nutrients (Secades, Alvarez & Guijarro 2003). The ability to degrade elastin has been suggested to be important for *F. psychrophilum* virulence, since it was at first found in all virulent strains (Madsen & Dalsgaard 1998). However, later studies found the ability in non-virulent strains as well (Madsen & Dalsgaard 2000). Furthermore, the two metalloproteases Fpp1 and Fpp2 are suggested to be responsible for the formation of narrow tubular holes found on fin rays of infected fish (Luis Martinez, Casado & Enriquez 2004).

Two distinct colony types have been described: Smooth and rough. Both are yellow due to flexirubin production (Jooste & Hugo 1999), but one type has regular edges, whilst the other has spreading margins. When grown in broth, cells of the smooth colony type auto-agglutinate at the bottom of the tubes, while cells of the rough colony type cells are non-agglutinating and uniform in growth (Bernardet & Kerouault 1989; Pacha 1968; Högfors-Rönnholm & Wiklund 2010). Both types are virulent and can be isolated from diseased fish. Outer membrane proteins and lipopolysaccharides were found to be similar in both the smooth and rough phenotype, but the smooth cells were more hydrophobic and more adhesive. However, both cell types were virulent in intramuscular challenge (Högfors-Rönnholm & Wiklund 2010).

[3] Movement of bacteria without the use of flagella is referred to as gliding motility. The mechanism in *F. psychrophilum* is unknown but pili have not been demonstrated in *F. psychrophilum* (Nematollahi, Decostere, Pasmans & Haesebrouck 2003) and a study of *Flavobacterium johnsoniae* makes it unlikely that polysaccharide secretion is involved (Hunnicutt & McBride 2000).

1.3. Cultivation and serology

Nutrient poor media, such as the tryptone yeast extract salts (TYES) agar or Cytophaga agar (Ordel's agar), must be used for cultivation of *Flavobacterium spp.* (Figure 1). When cultivated on TYES agar, colonies are bright yellow with a raised and shiny surface and sometimes surrounded by a thin spreading zone (Møller 2004). The bacteria will not grow on blood agar or other nutrient rich media. Growth has been reported at 5 - 23 °C and in 0 - 1 % NaCl (Holt *et al.* 1993), though optimal growth is achieved at 15 °C (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). Because of slow growth at higher temperatures and lack of growth at even higher temperatures, *F. psychrophilum* is considered to be psychrophilic. Colonies are most often bright yellow and glossy, raised on the plate and with a thin spreading edge (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). The detailed method used for cultivation in the present thesis is described in MMAH I.

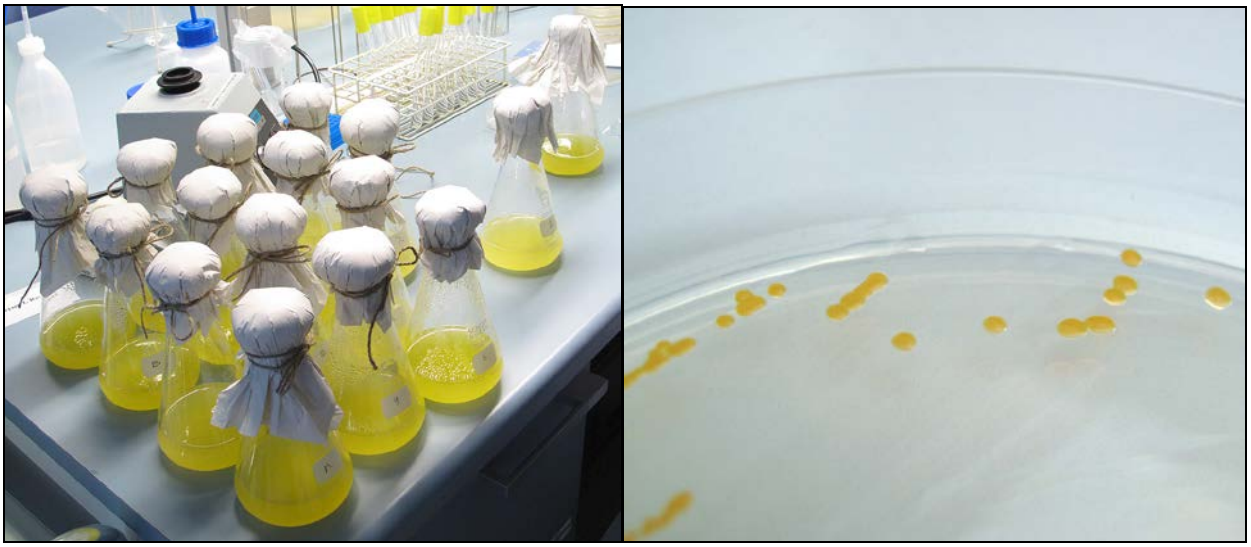


Figure 1. *F. psychrophilum* grown in TYES broth (left) **and on TYES agar plates** (right)**.**

Before carrying out experimental infections, the potential effect on cultivation of using plastic or glass tools for plate counting of *F. psychrophilum* CFU was examined. No difference was found, but a decrease in CFU was repeatedly observed after approximately 24 hours (data not shown), which was not reflected in the OD measurements (Figure 2). Generally, CFU counts were difficult to reproduce, especially for the 24 hour culture.

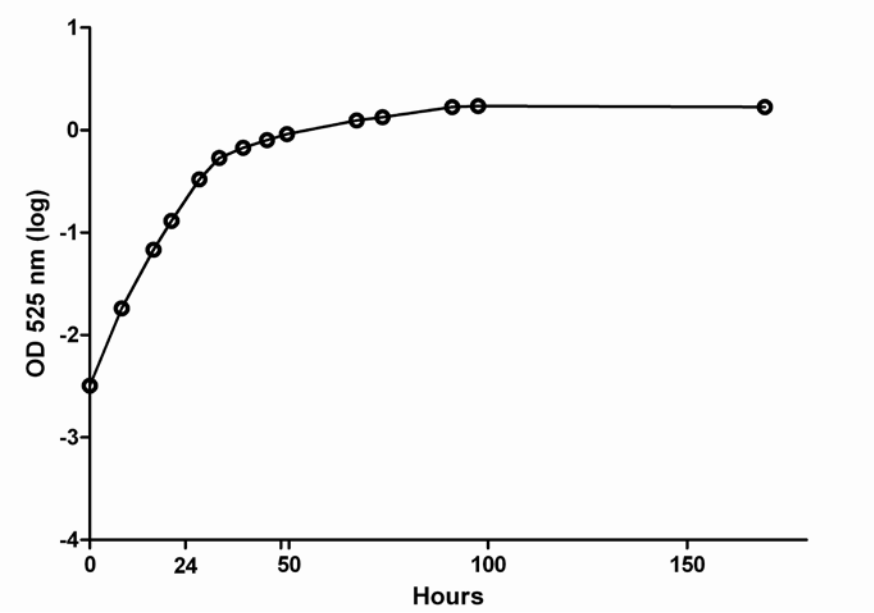


Figure 2. Growth curve for *F. psychrophilum* strain 950106-1/1. The depicted growth curve shows the mean values of three separate experiments done in duplicate.

Water samples were taken from each replicate tank after the rainbow trout fry had been exposed to H_2O_2 and *F. psychrophilum* grown for either 24 or 48 hours with or without prior exposure to H_2O_2 (Experiment 2 – MMAH I). The samples were incubated on TYA plates. CFU could not be quantified due to the appearance of several different colony types, but a visual assessment was carried out. For water samples from tanks where the 48 hour culture had been used for challenge, few or no culturable, yellow *F. psychrophilum*-like colonies were present after the fish had been immersed for 30 minutes. The results were not influenced by exposure to H_2O_2. *F. psychrophilum*-like colonies were re-isolated from the water from both groups immersed in the 24 hour culture, and in a higher number from the groups, where fish had been pre-treated with H_2O_2 (data not shown). A previous study showed an increased mortality in rainbow trout immersion-exposed to a *F. psychrophilum* culture grown for 24 hours, which corresponded to the logarithmic phase of growth (Aoki, Kondo, Kawai & Oshima 2005). Held together, these observations indicate that the capacity for adhesion of *F. psychrophilum* to the fish is influenced by growth phase. The results are not conclusive, and the subject should be investigated further, especially since the majority of experimental infections have been carried out using bacterial cultures grown for 48 hours, where the bacterium is in the late stage of logarithmic growth.

Brain, kidney and spleen were sampled from experimentally infected rainbow trout fry dying after immersion-challenge with *F. psychrophilum* with and without pre-treatment with 150 mg L^{-1} H_2O_2 (Experiment 3 - MMAH I). It was possible to re-isolate the bacteria from ≥72 % of all organs sampled. A significantly higher number of spleens from the *F. psychrophilum* group were positive (90 %), compared to the H_2O_2 + *F. psychrophilum* group (74 %) (MMAH III). Re-isolation of the pathogen in the gills was not done but would have been advantageous, since the pathogen load was

assessed by measuring the presence of genus specific 16S rRNA with qPCR and FISH was used to localize the bacteria.

Serotyping is one of the tools used to distinguish between different *F. psychrophilum* strains. Several studies regarding antigenic variation have been carried out. The first resulted in the division into three serotypes: Fd, Fp^T and Th (Lorenzen & Olesen 1997), where serotypes Fd and Th represent more than 88 % of the Danish isolates causing disease (Dalsgaard & Madsen 2000). Fp^T was not found to be associated with disease in rainbow trout, which has also been verified through experimental observation (Madsen & Dalsgaard 1999). Another study divided isolates into six distinguished serotypes and an untypeable serotype. Serotype O1[4] contained isolates affecting coho salmon while O2 affected ayu and O3 rainbow trout (Izumi & Wakabayashi 1999). Another characterization of seven host-specific serovars from worldwide isolates has also been reported (Mata, Skarmeta & Santos 2002). Thus, an international standardization regarding serotyping of *F. psychrophilum* strains does not currently exist, and more work is needed in order to achieve this.

1. 4. The disease RTFS / BCWD

RTFS outbreaks usually occur at water temperatures below 15 °C and may result in mortality rates ranging up to 90 % in fry, while larger fish display notably lower mortality rates (Nematollahi, Decostere, Pasmans & Haesebrouck 2003; Lorenzen 1994; Nilsen *et al.* 2011). The clinical symptoms vary, but typical characteristics of the disease (Figure 3) include lethargy, loss of appetite, dark coloration of the skin, accumulation of fluid in the peritoneal cavity, protrusion of the eyes, enlargement of the spleen, anemia manifested in pale gills and organs, atrophy of the anterior kidney and fragile and inflamed intestine. Skin lesions may appear in larger fish, typically on the peduncle or jaw and may in severe cases expose the underlying muscle (Nematollahi, Decostere, Pasmans & Haesebrouck 2003; Lorenzen *et al.* 1991; Bruno 1992).

Outbreaks of RTFS usually occur within the first two months of feeding, while the immune-system of the trout is not yet fully developed. Intraperitoneal injection with *F. psychrophilum* showed (Decostere *et al.* 2001), that fry weighing 1 g (aged 10 weeks) displayed clinical symptoms and suffered mortality, while this was not the case for fish weighing over 25 g (aged 20 weeks).

[4] Serotype O1 corresponds to FpT.

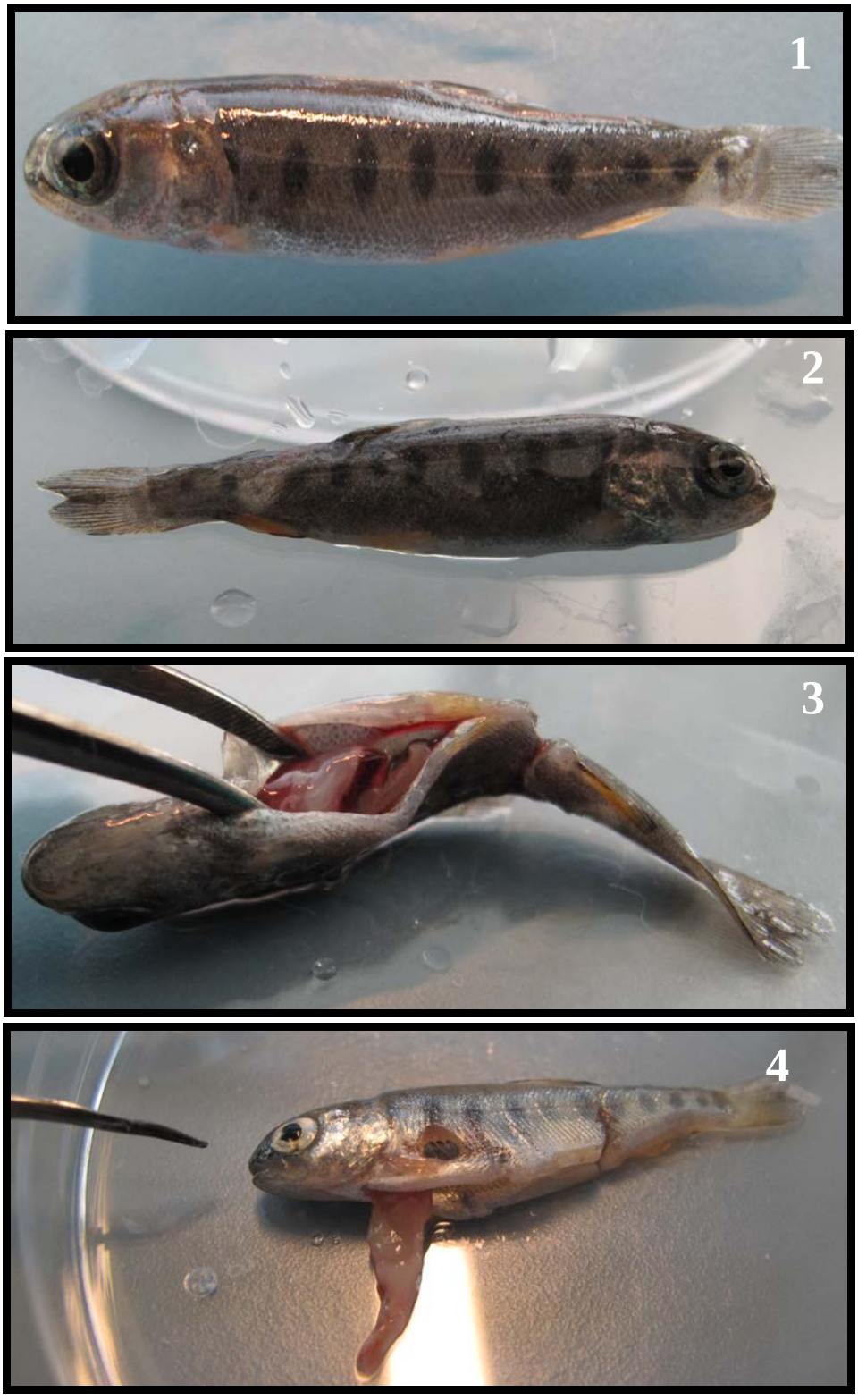


Figure 3. Clinical signs on fry experimentally challenged with *F. psychrophilum*. 1) A normal rainbow trout fry. 2) The clinical signs of RTFS are numerous and vary, but a dark coloration of the skin is often observed. 3) An enlarged spleen is common. 4) A severe skin lesion, which is most prevalent in larger fish and is rarely seen in fry.

The route of infection has not yet been established, though several hypotheses exist. Previous Danish studies regarding the occurrence of *F. psychrophilum* in fish farms have shown that the bacteria can be isolated from gills, skin, wounds and internal organs from both clinically and non-clinically affected fish (Dalsgaard & Madsen 2000; Madsen *et al.* 2005). Many different approaches for establishing a reproducible immersion-based experimental infection have been tried but success has been dependent on the skin being damaged prior to immersion (Madetoja, Nyman & Wiklund 2000). Adhesion of the bacteria to the gills has been suggested as an important first step for establishing of a successful experimental *F. psychrophilum* infection (Nematollahi, Decostere, Pasmans, Ducatelle & Haesebrouck 2003; Rangdale 1995).

Flavobacterium spp. are considered to be commensal bacteria; being present as a part of the normal microbial flora, and *F. psychrophilum* is therefore regarded as an opportunistic rather than obligate pathogen (Cahill 1990). *F. psychrophilum* cells are released into the water from dead fish at a high rate than from live fish after intraperitoneal injections (Madetoja *et al.* 2000). Furthermore, bacterial cells have been shown to survive for several months in stream water (Vatsos *et al.* 2003; Madetoja, Nystedt & Wiklund 2003). The virulence of starved bacteria droped over time, but even after starvation for ≈50 days, mortality rates following subcutaneous injections still reached 50 % (Madetoja *et al.* 2003). Viewed together, these features may help to explain reoccurring disease outbreaks.

1.4.1. Pathogenesis and virulence

The pathogenesis of RTFS is not yet understood and limited data on virulence factors is available. The virulence of different *F. psychrophilum* isolates vary extensively. Virulence factors include adhesins, exotoxins, endotoxins and proteases contributing to pathogenicity (Dalsgaard 1993). An association between the inability to cause disease for serotype Fp^T and its lacking ability to degrade elastin has previously been demonstrated (Madsen & Dalsgaard 1999).

For pathogenic bacteria, the ability to adhere to a potential host is a prerequisite for entrance and subsequently proliferation. Besides the adhesive interactions between pathogen and host cells mediated by hydrophobic, ion-bridging and receptor-ligand interactions, many bacterial structures can mediate adherence (Wilson, Henderson & McNab 2002). More virulent strains[5] of *F. psychrophilum* have been reported to adhere more readily to gills compared to less virulent strains, especially in water mixed with nitrite or organic material (Nematollahi, Decostere, Pasmans, Ducatelle *et al.* 2003).

F. psychrophilum is thought to be able to survive inside macrophages, especially in the spleen, which was also affected histopathologically after natural infection with *F. psychrophilum* (Decostere *et al.* 2001; Nematollahi, Pasmans, Haesebrouck & Decostere 2005; Rangdale, Richards & Alderman

[5] Two strains were used; Dubois was isolated from a Belgian RTFS-outbreak with 70 % mortality (Decostere, D'Haese, Lammens, Nelis & Haesebrouck 2001) and 99/10A, which was isolated from a healthy Danish rainbow trout (Madsen & Dalsgaard 1998).

1999). As previously mentioned, *F. psychrophilum* was re-isolated from a significantly higher number of spleens belonging to the *F. psychrophilum*, compared to the H_2O_2 + *F. psychrophilum* group (74 %) (MMAH III). Splenic macrophages have previously been demonstrated to be more tolerant towards harboring *F. psychrophilum* cells, thereby providing a place of safety inside the host (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). The spleen is responsible for filtering the blood and also has immunological functions. Recently, the presence of dendritic cells in the organ has been proposed (Lovy, Wright & Speare 2008). Thus, the presence of *F. psychrophilum* in the spleen may reflect both the bacteria's presence in the blood and removal by mechanical filtering or indicate initiation of the immune response. An intracellular strategy may protect the bacteria from lysozyme, complement and other humoral factors.

Serum of rainbow trout infected with *F. psychrophilum* has been shown to have a very high antibody level (Wiklund & Dalsgaard 2002; LaFrentz *et al.* 2002), but the number of bacterial cells only decreased slightly after incubation in immune serum. This suggests that specific antibodies themselves are not enough to confer protection against RTFS (Nematollahi, Decostere, Pasmans & Haesebrouck 2003; Wiklund & Dalsgaard 2002). The mode of action for antibodies against *F. psychrophilum* is still to be elucidated; they may either affect the bacterium directly or neutralize secreted cellular products, which play a part in pathogenesis.

Both virulent and avirulent *F. psychrophilum* strains have been demonstrated to activate the complement system *in vitro* in rainbow trout, although neither activation of the classical nor the alternative pathway affected survival of *F. psychrophilum* (Wiklund & Dalsgaard 2002). It has been speculated that the complement resistance may be due to the presence of LPS and a polysaccharide capsule, since such a connection has been demonstrated in other fish pathogens (Nematollahi, Decostere, Pasmans & Haesebrouck 2003).

The plasmid content in *F. psychrophilum* is reported to be very homogenous, with most strains carrying a 3.3 kb cryptic plasmid designated pCP1. A connection between the plasmid and virulence has not been shown (Madsen & Dalsgaard 2000), since it was present in both highly virulent and less virulent strains.

1.4.2. Prevention and control

Presently, no commercially available vaccine exists, although several attempts have been made (Högfors, Pullinen, Madetoja & Wiklund 2008; Obach & Laurencin 1991; Plant, LaPatra & Cain 2009; Dumetz *et al.* 2006; Lorenzen, Brudeseth, Wiklund & Lorenzen 2010). Consequently, antibiotics are currently the best treatment for RTFS, though the use has been subject to debate due to development of resistance (Dalsgaard & Madsen 2000; Schmidt, Bruun, Dalsgaard, Pedersen & Larsen 2000) and discharge into the surrounding environment. Besides vaccination, other prophylactic approaches against *F. psychrophilum* include good management practices and rearing environment. The application of stimulants in feed, prebiotic or probiotic feed additives may also be

considered. Additionally, disinfection of eggs has proven to be important to avoid vertical transfer from brood fish to fry (Madsen *et al.* 2005).

Conclusion

Flavobacterium psychrophilum is a Gram negative fish pathogen and the causative agent of RTFS/BCWD. Outbreaks of RTFS can result in high mortality rates and are usually seen in rainbow trout fry, while all salmonids are expected to be susceptible. No vaccine is currently available and the disease is treated with antibiotics. Cultivation of *F. psychrophilum* is problematic, since the bacterium is fastidious and does not grow on nutrient-rich media. The use of glass or plastic tools does not seem to influence CFU counts. Preliminary results indicate that the different stages of growth may affect the capacity of adherence for *F. psychrophilum* to a potential host. The bacterium has been isolated from both clinically and non-clinically affected fish and can survive under nutrient-poor conditions for a long time, which may explain reoccurring outbreaks. The route of infection and defense mechanisms in the host are still unclear. More work is needed to elucidate the portal(s) of entry, standardize the different serotypes and elucidate pathogenesis in future studies.

2. Experimental infections

Reliable experimental models are needed to gain understanding of infectious diseases. Injection-based experimental challenges with *F. psychrophilum* have been standardized and the resulting mortality depends on several factors, including the choice of bacterial strain, number of colony forming units (CFU), administration of the bacteria, size of the fish, batch differences and the number of fish in each tank (Madsen & Dalsgaard 1999). However, infection by injection is not a suitable approach for all investigations, since the first line of defense, consisting of mucus, skin, gill and gut, is bypassed. Two approaches are used for infection of fish without injecting the pathogen into the host. In immersion models, the fish are bathed in a high bacterial suspension. In cohabitation, donor fish are injected with the pathogen and marked, e.g. by fin clipping, and placed in a tank with healthy recipients.

Several studies have focused on the development of reproducible experimental models for RTFS infection without the use of injection. It has generally proven difficult to produce high and consistent mortality rates using immersion and cohabitation, unless stress or scarification has been applied. However, high mortality rates have been reported in some studies (Table 2). Immersion exposure to *F. psychrophilum* in the logarithmic phase of growth resulted in high mortality rates in rainbow trout at 17 °C (Aoki *et al.* 2005). The strain NCIMB1947 was used in the experiment, which was isolated from a kidney of a Coho salmon in the USA. The strain belongs to serotype Fp^T (O1), which is not associated with disease in rainbow trout. The bacterium was passed through rainbow trout 5 times before being used in challenge. A mortality rate of 31.5 % was reported after immersion of 1.3 g fish in an 18 hour culture for 60 minutes, 58.2 % in a 24 hour culture and 6.9 % for a 48 hour culture. The 18 and 24 hours cultures corresponded to early and late phase of logarithmic growth, respectively. The results from the 24 hour culture were not reproducible using the Danish strain 950106-1/1 at 14.5 ±0.5 °C in a small-scale study on 0.8 g fry performed as a part of the present thesis (Experiment 2 - MMAH I). Strain 950106-1/1 is virulent and belongs to serotype Fd and was isolated from a rainbow trout during a clinical outbreak in a Danish freshwater farm in 1995 (Madsen & Dalsgaard 1999). Exposure to 0.005 % formalin before immersion of 1.0 g rainbow trout in strain 950106-1/1 was reported to elevate mortality from 27 - 31 % to 41 - 66 % at 12 °C, although potential mortality from formalin alone was not investigated (Madsen & Dalsgaard 1999). Immersion in the Finnish strain T1-1 has been reported to elevate mortality from 0 % in intact fish to 95 % in 0.4 g fish with skin wounds at 11.5 ±0.5 °C. A similar pattern was observed in a cohabitation challenge, where mortality was increased from ≤ 10 % in intact recipients to 64 % in recipients weighing 1.0 g with skin wounds (Madetoja *et al.* 2000). High mortality rates in 5.8 g fish have also been reported after immersion in the French strain JIP P29-98 at 10 °C, and mortality was increased by inflicting lesions, stressing the fish by minimizing the water volume and replacing water with 0.9 % NaCl. However, mortality in the control group was 38.3 % and mortality between the groups was not compared statistically, so it is unknown whether the various treatments significantly increased mortality (Garcia *et al.* 2000). Other experimental infections have resulted in mortality after prolonged exposure to *F. psychrophilum* for 5 and 10 hours, but it was concluded that the method was not reliable (Rangdale

1995; Holt 1987). Other approaches for experimental infection have been tried. Concomitant exposure to the parasite (*Gyrodactylus derjavini*) did not result in mortality (Busch, Dalsgaard & Buchmann 2003), and oral and anal challenges have also been carried out without resulting in mortality using four different strains (Decostere *et al.* 2000; Madetoja *et al.* 2000). Some previous experiments have used small experimental groups and few or no replicates (Table 2) and followingly, the results may not be fully conclusive.

Due to the absence of a reliable model for infection which imitates natural transmission, information regarding the first phase of infection and in particular interactions between the host and *F. psychrophilum* are limited. This can largely be attributed to the lack of a reproducible immersion-challenge method (Decostere *et al.* 2000), which has led to the use of naturally infected and injection-challenged fish in studies regarding the immune response (Evenhuis & Cleveland 2012; Orieux, Douet, Le Henaff & Bourdineaud 2013; Overturf & LaPatra 2006; Villarroel *et al.* 2008). Furthermore, these studies have been focused on larger fish (10 – 50 g), even though RTFS is most severe in fry. Since natural transmission is mimicked in an immersion-based model, the approach is useful in investigations concerning the host immune response. Even though the portal(s) of entry are yet unidentified, *F. psychrophilum* has been found in mucus, fins, gills and stomach of infected fish. This has led to speculations as to the role of sub-optimal environmental conditions which may allow the bacterium to get across skin and gills (Nematollahi, Decostere, Pasmans & Haesebrouck 2003).

Bath-treatment with various non-medical compounds, such as copper sulphate, chloramine-T, sodium carbonates, sodium chloride, formalin and H_2O_2, are routinely used against various pathogens in aquaculture (Jokumsen & Svendsen 2010). Exposure to formalin before immersion in *F. psychrophilum* has previously been used to elevate mortality (Madsen & Dalsgaard 1999). The use of formalin is to be phased out by Danish fish farmers due to human health considerations, resulting in the need for a less harmful stressor. H_2O_2 is considered to be environmentally friendly since it is broken down to water and oxygen, and has been suggested as an alternative to formalin in several regards (Pedersen 2010; Sortkjær *et al.* 2000). Furthermore, the use of H_2O_2 has been shown to accelerate *Tenacibaculum maritimum* infections in turbot (Avendaño-Herrera, Magariños, Irgang & Toranzo 2006) and *Flavobacterium columnaris* infections in channel catfish (Thomas-Jinu & Goodwin 2004). Accordingly, H_2O_2 was an obvious candidate for pre-treatment in a new *F. psychrophilum* immersion model (MMAH I).
The experimental setup included four groups: 1) Control, 2) H_2O_2, 3) *F. psychrophilum* and 4) H_2O_2 + *F. psychrophilum* (Figure 4). Experiment 3 showed that pre-treatment with H_2O_2 significantly elevated the mortality of subsequent immersion-exposure to *F. psychrophilum*. Based on the results, the experimental setup was optimized in Experiment 4. Firstly, handling of replicates was uniformed in hopes of lowering variation. Secondly, the number of replicates in the infected groups was increased from two to three. Alternatively, the size of the groups could have been increased, but this was not possible since the available tanks could hold no more than ≈50 fish. Thirdly, the temperature was lowered to delay the immune response in the fish (Raida & Buchmann 2007) and give the psychrophilic bacteria an advantage. Finally, H_2O_2 pre-treatment dosage was increased from 150 mg L^{-1} to 200 mg L^{-1}. Although variation in the infected groups remained unchanged, the mortality in

both infected groups was increased. In Experiment 3, pre-treatment with H_2O_2 increased mortality of subsequent exposure to *F. psychrophilum* from 9.1 % to 19.2 %, while the increase was from 14.7 % to 30.3 % in Experiment 4. Thus, the increase in mortality due to H_2O_2 exposure prior to immersion in *F. psychrophilum* was reproducible.

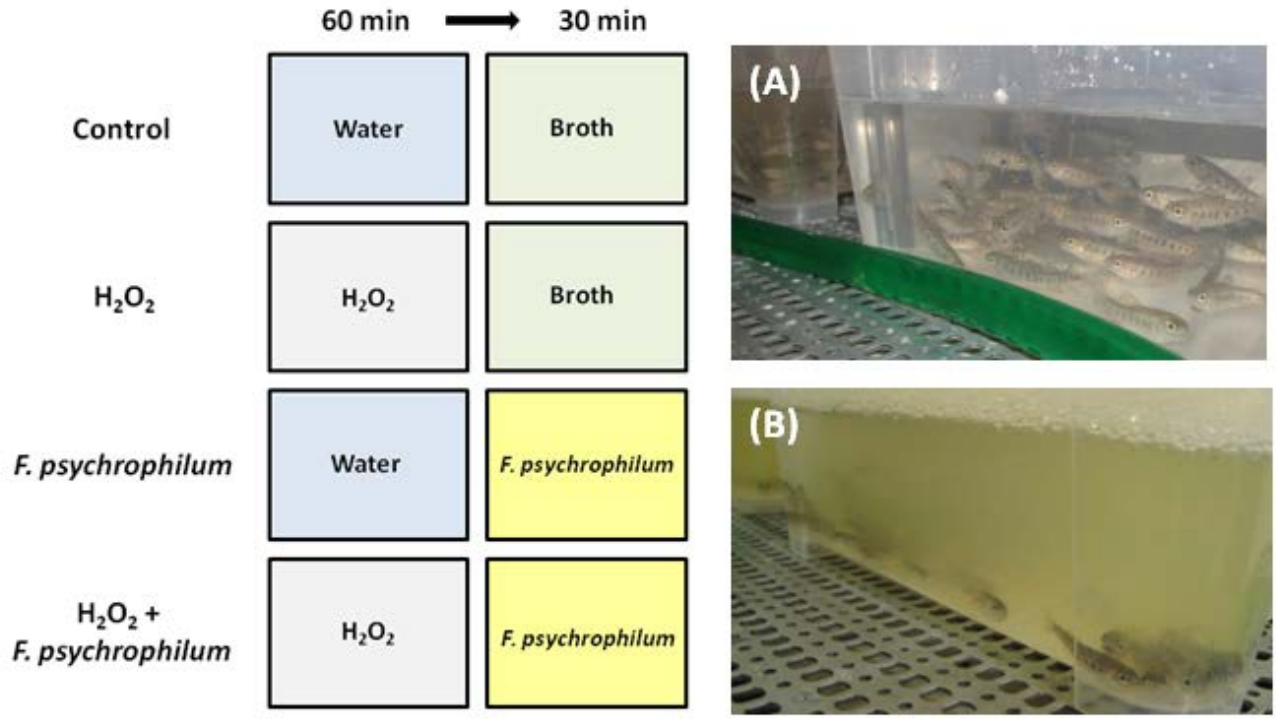


Figure 4. Overview of the experimental design. Left: Overview of the four groups included in the H_2O_2-immersion model. Fish not treated with H_2O_2 were instead placed in tanks with water and fish not infected with *F. psychrophilum* were immersed in a 1:9 diluted sterile broth. Right: (A) shows the fish in before exposure and (B) shows the fish immersed in the 1:9 diluted bacterial broth.

Conclusion

It has been difficult to induce mortality with *F. psychrophilum* in an experimental setting and it is unlikely that immersion-challenge will ever be standardized to the same level as injections. Experimental immersion-based challenges have rarely resulted in mortality without either compromising the skin or mucus layer or by exposing the fish to formalin or H_2O_2 before challenge. However, high and reproducible mortality rates after exposure to *F. psychrophilum* in the logarithmic phase of growth have been reported, although the results were not reproducible in the present thesis (Experiment 2 – MMAH I). The underlying cause for the increased mortality after exposure to formalin and H_2O_2 may be due to skin or mucus damage, since H_2O_2 is known to damage the gills (Tort *et al.* 2002) and formalin affects epithelial structure and mucous cell density (Buchmann, Bresciani & Jappe 2004). Alternatively, the increased mortality may be caused by stress due to the exposure. This question needs to be clarified, e.g. by demonstrating entry into the host through the damaged skin or gills after exposure. Despite a high variation, the elevation of mortality in the model

using H_2O_2 is reproducible and hence seems to be a good alternative to injections, when emulation of natural transmission is favored. If the most favorable conditions for *F. psychrophilum* were identified, the application of H_2O_2 might allow for further increased mortality. Ideally, the model would produce 60 – 80 % mortality and be applicable to the testing of vaccines (Amend 1981).

Table 2. Overview of immersion and cohabitation based experimental infections with *F. psychrophilum*. Immersion-based experimental infections with *F. psychrophilum* have rarely resulted in mortality without application of scarification or pre-treatment with either of the non-medicine therapeutical compounds formalin and H_2O_2. Additionally, prolonged immersion has resulted in mortality without damages to the skin, but the method was deemed unreliable (Rangdale 1995; Holt 1987).

Method		Strains	CFU	°C	n	Fish size	Mortality	Reference
Immersion with H_2O_2 as stress	- 150 mg L^{-1} (60 min)	960106-1/1	10^7 mL^{-1} (30 min)	16.8 ±0.5	≈50x2	1.2 g	9.1 % 19.2 %[1]	(MMAH I)
	- 200 mg L^{-1} (60 min)	960106-1/1	10^7 mL^{-1} (30 min)	14.0 ±0.5	≈50x3	1.1 g	14.7 % 30.2 %[1]	(MMAH I)

[1] Pre-treatment with H_2O_2 was reported to significantly increase mortality in both experiments. Mortality in the unstressed group was also significantly larger compared to the control groups.

Immersion with two bacterial growth stages (24/48 h)	- 200 mg L^{-1} H_2O_2 (60 min)	24 h 960106-1/1	10^5 mL^{-1} (30 min)	14.5 ±0.5	20 12	0.8 g	15 % 25 %	(MMAH I)
	- 200 mg L^{-1} H_2O_2 (60 min)	48 h 960106-1/1	10^7 mL^{-1} (30 min)		20 19		5 % 21 %	(MMAH I)

Immersion with various *F. psychrophilum* strains		32/97, JIP 30/98, B97034 E4, B97026 P1, Dubois, OSU 84-254, UCD 004-95, SvS 910611-1, FPC 840, LVDI 5/I	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min) 10^8 mL^{-1} (45 min)	10	10x20 10x20 10x20	1.5 g	0 % 0 % 0 %	(Decostere *et al.* 2000)
Immersion at 15 °C		Dubois, B97034 E4, SVS 910611-1	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min) 10^8 mL^{-1} (45 min)	15	3x20 3x20 3x20	1.5 g 1.5 g 1.5 g	0 % 0 % 0 %	(Decostere *et al.* 2000)
Immersion and scarification	Skin[2] Gills[2]	Dubois, B97034 E4, SVS 910611-1	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min)	10	3x20 3x20	1.5 g	0 % 0 %	(Decostere *et al.* 2000)
Immersion and water quality	0.15 mg L^{-1} NH_3 0.25 mg L^{-1} NO_2 Formulated water[3]	Dubois, B97034 E4, SVS 910611-1	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min) 10^8 mL^{-1} (45 min)	15	3x20 3x20 3x20	1.5 g	0 % 0 % 0 %	(Decostere *et al.* 2000)

[2] The skin was wounded using a 26G needle and the gills were damaged by clipping.

[3] Distilled water supplemented with 0.03 % NaCl, 0.01 % KCl, 0.002 % $CaCl_2 2H_2O$ and 0.004 % $MgCl_2 6H_2O$.

Immersion in bacteria grown in different culture mediums	MAOB[4], TYE[4], CAC[4], SDI[4]	Dubois, B97034 E4, SVS 910611-1	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min) 10^8 mL^{-1} (45 min)	10	4x20 4x20 4x20	1.5 g	0 % 0 % 0 %	(Decostere *et al.* 2000)

[4] MAOB: modified Anacker and Ordal broth; TYE: tryptone yeast extract salts broth; CAC: cytophaga broth containing various carbohydrates; SDI: Shieh broth devoid of iron and supplemented with 2·2-dipyridyl.

Immersion with stress (0.0005 % formalin for 30 min)	Starvation Handling Continuous light	Dubois, B97034 E4, SVS 910611-1	10^6 mL^{-1} (45 min) 10^7 mL^{-1} (45 min) 10^8 mL^{-1} (45 min)	10	4x20 4x20 4x20	1.5 g	0 % 0 % 0 %	(Decostere *et al.* 2000)

Immersion		911209-1	10^8 mL^{-1} (50 min)	10.5 ±0.5	2x60	1.3 g	0 %	(Lorenzen, Brudeseth, Wiklund & Lorenzen 2010)

Immersion combined with a parasite (*Gyrodactulus derjavini*)	*F. psychrophilum* *G. derjavini* *G. derjavini* + *F. psychophilum*	950106-1/1 - 950106-1/1	10^7 mL^{-1} (30 min)	12.5 ±0.5	2x25 2x25 2x25	0.8 - 2.5 g	0 % 22 % 12-36 %	(Busch *et al.* 2003)

Immersion combined with a parasite (*Gyrodactulus derjavini*)	*F. psychrophilum*	950106-1/1	10^7 mL^{-1} (10 h)	12.5 ±0.5	2x25	0.8 - 2.5 g	0 %	(Busch *et al.* 2003)
	G. derjavini	-			2x25		17 %	
	G. derjavini + *F. psychophilum*	950106-1/1			2x25		8-12 %	

Immersion in various bacterial growth stages	18 h	NCMB1947	10^7 mL^{-1} (60 min)	17	108	1.3 g	31.5 % [5]	(Aoki *et al.* 2005)
					117	5.6 g	35.0 % [5]	
	24 h		10^7 mL^{-1} (60 min)		91	1.3 g	58.2 % [5]	
					82	5.6 g	37.8 % [5]	
	48 h		10^8 mL^{-1} (60 min)		101	1.3 g	0 %	
					87	5.6 g	6.9 % [5]	
	66 h		10^6 mL^{-1} (60 min)		105	1.3 g	0 %	
					108	5.6 g	0 %	

[5] All groups with mortality were reported to have a significantly increased mortality compared to the control group (with no mortality).

Immersion with formalin as stressor	-	950106-1/1	10^7 mL^{-1} for 30 min	12	≈2x50	1.0 g	27-31 % [6]	(Madsen & Dalsgaard 1999)
	0.005 % formalin for 30 min				≈2x50		41-66 % [6]	

[6] Significance levels were not reported.

Cohabitation[7]	Cohabitants	950106-1/1	10^4	12	4x25 (50)	1.0 g	8-16 %[8]	(Madsen & Dalsgaard 1999)
	I.p. injected				4x25 (50)		80-100 %[8]	

[7] Half the group i.p. infected and the other half was marked by clipping in adipose fin.
[8] Significance levels were not reported.

Immersion; stress, lesions and salinity	-	JIP P29-98	10^6 for 60 min	10	3x20	5.8 g	38.3	(Garcia *et al.* 2000)
	0.9 % NaCl				3x20		40	
	Stress[9] (4)				3x20		50	
	Stress[9] + 0.9 % NaCl				3x20		46.7	
	Lesion[10]				3x20		55	
	Lesion[10] + 0.9 % NaCl				3x20		46.7	

[9] The fish were stressed by being kept for 60 min in minimal water volume.
[10] Lesions were created by fin ablation.

Immersion with compromised skin	Intact	T1-1	10^6 for 60 min	11.5 ±0.5	2x20	0.4±0.1 g	0 %	(Madetoja *et al.* 2000)
	Skin wounded				≈2x20		95 %	
	Non-intact skin mucus				≈2x20		27 %	

Cohabitation[11] with compromised skin	Intact	T1-1	10^5	11.5 ±0.5	2x15 (2x5)	1.0±0.2 g	≤10 %[12] (100 %)[13]	(Madetoja *et al.* 2000)
	Skin wounded				2x15 (2x5)		64 %[12] (80 %)[13]	
	Non-intact skin mucus				2x15 (2x5)		≤10 %[12] (90 %)[13]	

[11] Five fish pr. tank were subcutaneously injected donors.
[12] Mortality rates were only significant in the 'skin wounded' group; mortality rates for 'intact' and 'non-intact skin mucus' were only provided in figures.
[13] Mortality rates for the donor fish are given in parenthesis.

Injection compared to retrobulbar scraping[12]	I.m. injected	10^7	1x10	100 %	(Ostland, McGrogan & Ferguson 1997)
	I.p. injected	10^7	1x10	100 %	
	Retrobulbar scraping	≈10^5 mL^{-1} for 30 min	1x10	0 %	

[12]The dorsal aspect of the retrobulbar region of the left eye was scraped five times with using a scalpel

3. The fish immune system

Most of the knowledge regarding function, regulation and composition of the immune system is based on studies in mice and humans, although interest in fish immunology has increased in recent years. Besides being important for aquaculture, other fields have begun to focus on fish as well, e.g. on communication between the so-called innate and adaptive parts and the evolutionary development of the immune system has received recent attention (Lieschke & Trede 2009; Magnadottir 2010).

Although fish are a paraphyletic[6] group, components of the innate immune system are phylogenetically indigenous and also present in evolutionarily primitive diploblastic metazoans: The phyla Ctenophora, Porifera and Cnidaria[7]. These possess phagocytic amoebocytes, which function as both digestive and defensive cells against invading organisms, and are the functional precursor of macrophages present in vertebrates (Lieschke & Trede 2009). The appearance of adaptive elements has long been considered to have appeared after Agnatha[8] on the evolutionary timeline. However, more recent studies demonstrated lymphocyte-like cells within Agnatha with accumulating evidence for immunological functions[9]. Gene rearrangement has been demonstrated in lampreys, although the mechanism differs from what is seen in Gnathostomata, the jawed vertebrates (Lieschke & Trede 2009). These findings suggest the presence of lymphocytes prior to the acquisition of adaptive immunity as we know it in teleosts.

The elements comprising adaptive immunity as they occur in higher vertebrates arose for the first time 400-500 million years ago in Gnathostomata. The thymus emerged, along with the recombination activating gene (RAG) family, the rearranging T cell receptor and rearranging immunoglobulin genes. These components allow immune responses to a virtually unlimited range of antigens. The structure of the T cell receptor is relatively constant through evolution, while immunoglobulin (B cell receptor when membrane-bound) show more variation (Lieschke & Trede 2009).

Blood is pumped by the heart and passes the gills while circulating systemically, before returning to the heart. Unlike in mammals, fish blood is oxygenated in the gills instead of the lungs. The layer of cells lining the heart chambers is highly phagocytic in some fish species, such as Atlantic cod (*Gadus morhua*). In salmonids, comparable cells are found in renal portal sinusoids in the kidney. These cells are also capable of detoxification through cytochrome P450, which catalyzes oxidation of organic substances. This suggest a role in monitoring of systemic diseases and to some extent the

[6] Paraphyletic groups consist of all descendants from the same common ancestor with the exception of a few monophyletic groups. In the case of fish, the superclass Tetrapoda (four-limbed vertebrates) is excluded.
[7] Comb jellies, sponges, corals and jellyfish.
[8] Jawless fish, including lampreys and hagfish.
[9] Opposed to the immunoglobulin-based receptors in jawed vertebrates, lampreys immune system recognize antigens via variable lymphocyte receptors (VLR) comprised of leucine-rich-repeats (LRR) and an invariant region. The potential repertoire of these receptors is estimated to be similar to what is seen in mammals (Guo *et al.* 2009).

regulation hereof (Ferguson 2006). Conclusive evidence for a secondary circulatory system in fish, corresponding to the lymphatic system in mammals, has only been published recently. A study in zebrafish found a secondary circulatory system, which was morphologically, molecularly and functionally distinct from blood vessels (Küchler *et al.* 2006).

Teleost fish have leukocytes comparable but not identical in shape and function to the macrophages, neutrophils, monocytes, thrombocytes, eosinophils, B cells, plasma cells, T cells and natural killer cells found in mammals. Fish B cells have been shown to have strong phagocytic and microbicidal abilities, ingesting injected bacteria and beads, and may thus not only be involved in humoral immunity (Li *et al.* 2006).

3.1. Lymphoid organs

The lymphoid organs of vertebrates are classified as either primary or secondary according to ontogeny and function. The primary lymphoid organs in fish include the head kidney, thymus and spleen, while secondary lymphoid organs include mucosa-associated lymphoid tissues.

3.1.1. Kidney

The primary site for hematopoiesis in teleosts is the anterior kidney (Fange 1986), and not the bone marrow as in mammals. Gross morphology of the kidney is subject to variation between species. Generally, the anterior part of the kidney has no renal functions and consists primarily of hematopoietic and lymphoid tissue with few if any renal tubules, and vice versa in the posterior kidney (Ferguson 2006; Zapata & Cooper 1990). The anterior kidney is also suggested to harbor dendritic cells (Lovy *et al.* 2008) and to be the site for B cell subset maturation (instead of the lymph nodes in mammals), after which they migrate to sites of activation (Zwollo, Cole, Bromage & Kaattari 2005).
A nephron constitutes the basic structural and functional component of the kidney, which filters the blood to form urine. While most excretory functions are maintained by the gills in freshwater fish[10], the kidney's role is to counteract the passive fluid influx over the gills by producing urine. Another distinct feature regarding the teleost kidney is the reticuloendothelial system: A system of macrophages, which separate and up-concentrate phagocytosed materials (Roberts 1989). Since teleosts lack lymph nodes, the kidney is one of the two major filtering organs, the spleen being the other (Ferguson 2006). The trout kidney is thus a complex organ with many functions.

3.1.2. Spleen

The spleen is found in virtually all vertebrates and serves several functions. The mechanical functions include filtering of the blood, which removes antibody-covered bacterial cells and

[10] Freshwater fish actively take up ions (Na^+, K^+, Cl^-) from the surrounding water. Water diffuses into the fish and is expelled as urine. Marine fish have a lower internal concentration of salts compared to the water and secrete salt from the gills and through urine.

senescent red blood cells. The spleen is also involved in the recycling of iron and contains a large amount of monocytes, which can differentiate into macrophages upon activation. Furthermore, the spleen has immunological functions, and is a key source of antibody production and immunological memory in rainbow trout, but has not been proven to have a role in the production of red blood cells (erythropoiesis) in adult trout (Ferguson 2006). The presence of dendritic cells has also been suggested (Lovy *et al.* 2008). A positive correlation between spleen size and resistance to *F. psychrophilum* has been documented. Whether this is due to greater filtering capacity or improved immune functions is unknown (Hadidi, Glenney, Welch, Silverstein & Wiens 2008).

3.1.3. Thymus

The thymus is located near the opercular cavity in teleosts and is involved in the maturation of T cells. The thymus is located superficially and is covered with pores, making it even more exposed to antigens from the surrounding environment (Chettri *et al.* 2012; Ferguson 2006).

3.1.4. Mucosa-associated lymphoid tissue

Living in an aquatic environment entails a constant possibility of exposure to various pathogens, at times in very high concentrations. The primary boundaries between fish and their surrounding environment are the mucosal surfaces and include the epithelia and associated tissues in gills, skin, gut, and the reproductive tract. These serve as primary sites of entry for most pathogens.
The first barriers encountered by pathogens are mechanical and include the dermis, epidermis, scales and mucus layer, which must be passed or breached in order to establish an infection. Mucus is constantly being produced and sloughed, which allows physical trapping of bacteria and thereby preventing them from reaching the tissue. In addition to the mechanical obstruction provided, mucus also provides a chemical barrier due to the content of antibacterial substances, including antimicrobial peptides, complement factors and immunoglobulin M (Magnadottir 2010; Ellis 2001). The internal epithelia combined with an environment consisting of acid, bile salts and enzymes protect the gastro-intestinal tract against pathogens.
In most cases the epithelial barriers are enough to stop invading microorganisms. If the epithelium is penetrated, inducible cellular and humoral innate defense mechanisms are activated (Magnadottir 2006). This enables the formation of memory cells, resulting in a faster and greater response to later infections with the same pathogen.

The gills

The gills are of great interest in the present thesis, since exposure to H_2O_2 has been shown to have no effect on skin, muscle, esophagus, liver, anterior and posterior kidney and spleen (Tort *et al.* 2002). The gills are one of the mucosal barriers in constant contact with the surrounding environment and considered to be an important portal of entry for several bacterial fish pathogens, e.g. the bacterial fish pathogen *Yersinia ruckeri* (Tobback *et al.* 2009; Ohtani & Raida 2013), although another study has shown that sideline, fins and rectum play significant roles (Khimmakthong *et al.* 2013). Recent research also point to the gills as an important organ regarding infection with

Aeromonas salmonicida and *Vibrio anguillarum* (Jayasuriya *et al.* 2013; Kato, Takano, Sakai, Matsuyama & Nakayasu 2013). Highly virulent strains of *Flavobacterium columnare* have been demonstrated to form biofilm-like structures in the gills of carp (*Cyprinus carpio*), where they destroy the tissue (Declercq *et al.* 2013). Finally, adhesion capacity to the gills and virulence of *F. psychrophilum* has been demonstrated to be correlated in a perfusion model in rainbow trout (Nematollahi, Decostere, Pasmans, Ducatelle *et al.* 2003).

In most species, each pair of gills consists of four arches on which the rows of primary lamellae are located. The feathery secondary lamellae (Figure 5) are located on the primary lamellae and are the site for gaseous exchange, when water is pumped over the gills counter-currently to the blood flow. Each secondary lamella can be perceived as a thin envelope over a central row of pillar cell. Each respiratory surface is comprised of a double layer of epithelial cells, which are joined by their margins. Migrating inflammatory cells are found in the interstitial space between these two layers. Diffusion efficiency depends on the distance between lamellar surface and blood in the pillar channels. Many types of gill diseases can increase the lamellar thickness by expanding intracellular spaces with fluid, inducing cellular hypertrophy[11] or hyperplasia[12], and thereby increasing the diffusion distance. Mucus production is increased when the gills are irritated by pathogens, pollutants or changes in water quality (Ferguson 2006; Wilson & Laurent 2002).

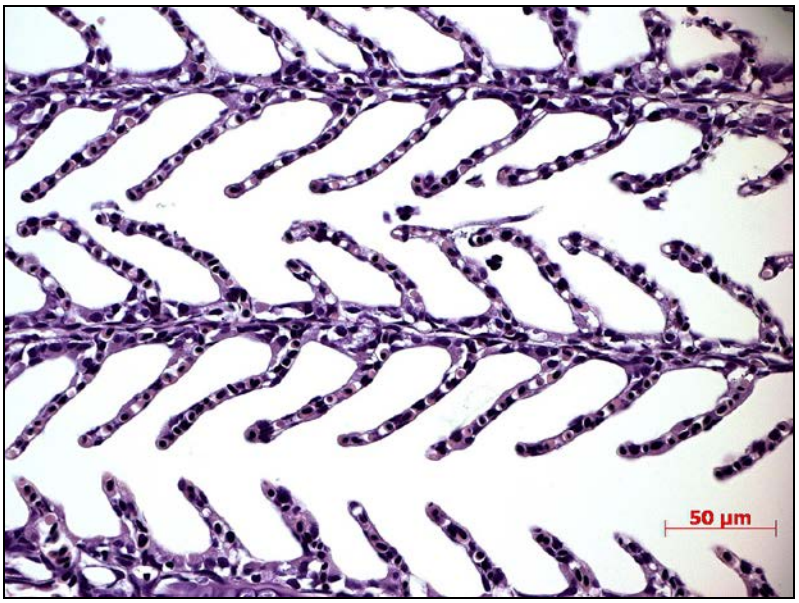


Figure 5. Structure of a normal rainbow trout gill. The secondary lamellae are located on the larger primary lamella.

Growing evidence suggest that pillar cells are a part of the reticuloendothelial system[13], and that they are to some extent analogous to intravascular pulmonary macrophages in mammalian lungs. Pillar cells can take up carbon and are often colonized by intracellular pathogens. Other similarities include

[11] Hypertrophy: increased cell size.
[12] Hyperplasia: increased number of cells.
[13] The reticuloendothelial system is also known as the macrophage system or the mononuclear phagocyte system, since the cells consists mainly of macrophages and monocytes.

uptake of foreign matter, shared antigenic profile with leukocytes and a high content of lysozyme. Rainbow trout gills have been shown to generate certain inflammatory mediators, but the implicated cells are yet unknown. Furthermore, pillar cells may have a gate-keeping function by taking up antigens and transferring them to resident macrophages located deeper in the brachial tissue (Ferguson 2006). Recent studies have found IgT positive cells in the epithelial lining of gills and CD8 positive cells at the base of the primary gill filaments, enabling the fish to respond quickly to invading pathogens. In younger life stages, where immunity is not yet fully developed, the dense layer of IgT may act as a shield against invading pathogens (Chettri *et al.* 2012; Olsen *et al.* 2011).

Goblet cells, osmoregulatory chloride cells and associated accessory cells are particularly present at the base of the lamella. Under pathological conditions, they are found in increasing numbers along the length of the lamellae. This is presumably a protective response to limit pathogen access to underlying epithelium or to enhance clearance of foreign particles. Other cells in the filamental interstitium include macrophages, eosinophilic granular cells, neuroepithelial cells and rodlet cells (Ferguson 2006; Wilson & Laurent 2002). Also, cells resembling dendritic cells have been found, supporting the hypothesis that gills play an important role in immune surveillance through antigen recognition, uptake and processing (Lovy *et al.* 2008).

3.2. Principles of innate immunity

The innate immune system elicits immediate action in the first period after infection, and innate immunity is of prime importance to fish (Magnadottir 2010). All higher vertebrates have similar immune systems, which are classically divided into two main parts: The innate and less specific part, which generally precedes the second part, which is the adaptive and acquired part. Despite this division, it is important to emphasize that both parts must interact in order to protect an organism against potential invaders (Magnadottir 2006). Commonly, innate immunity is further subdivided into three parts: epithelial/mucosal barriers, humoral parameters and cellular components (Magnadottir 2010).

3.2.1. Cells in innate immunity

The most important cells in the first line of defense in fish are the phagocytes, including neutrophils and monocytes/macrophages, and the non-specific cytotoxic cells (Magnadottir 2006). The latter is believed to be the pre-cursor for the natural killer cells found in mammals. Furthermore, epithelial and dendritic cells also contribute to the innate immune response. The innate immune system is also crucial to the activation of the adaptive immune system. Antigen-presenting cells engulf the pathogens and kill them in the phagolysosomes. Parts of the destroyed microbe are then transported to the phagocyte surface, where they are presented on MHC II to T cells, thereby initiating the adaptive immune response.

3.2.2. Recognizing the threat

Two categories of molecular patterns are believed to produce an immune response: Pathogen-associated molecular patterns (PAMP) and danger signals released during injury. The highly conserved PAMPs are, as previously mentioned, used for pathogen recognition by cells associated with innate immunity. The structures are invariant structures and universally found in pathogens and foreign materials and are not expressed by multicellular organisms. Well-known examples include lipopolysaccharides (LPS) found in the cell wall of Gram-negative bacteria, fungal β-glucan, viral double stranded RNA and unmethylated CpG motifs found in bacterial DNA (Magnadottir 2006). Danger-associated molecular patterns (DAMPs), on the other hand, are exposed through damage of host tissue. Damage can be caused by infection, necrosis or cell death and the recognized molecular patterns warn of danger.

Both DAMPs and PAMPs are recognized by pattern recognition receptors (PRRs). They are found on numerous cells in the immune system, including the surface epithelial cells, which are among the first to encounter invaders. The membrane-bound PRRs may be divided into two sub-groups based on function: Endocytic PRRs and secreted signaling PRRs.
The endocytic PRRs are found on the surface of phagocytic cells and include, among others, mannose receptors, scavenger receptors and opsonin receptors. They promote attachment, engulfment and destruction of microorganisms with the recognized pattern but do not transmit an intracellular signal. Signaling PRRs are also known as toll-like receptors (TLRs) and play a major part in activating both the innate and adaptive immune systems. At least 16 TLRs have been identified in teleost fish, but direct evidence of ligand specificity has only been shown for a few of them: TLR2, TLR3, TLR5M, TLR5S and TLR22 (Table 3). Although many similarities are seen with mammalian TLRs, non-mammalian and TLRs unique to teleosts also exist (Palti 2011). Some TLRs are found on the cell surface, while others are located intracellularly in the membrane of endosomes. Activation leads to the release of signaling molecules. Pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-12 and IL-8, trigger inflammation, fever and phagocytosis. Another group of cytokines, known as chemokines, enable migration of leukocytes from the blood to the inflamed tissue (Murphy, Travers & Walport 2008).
Secreted PRRs are released from the cell, which produces them, and include e.g. serum amyloid, C-reactive protein (CRP) and mannan-binding lectin (MBL).

	Mammals		Fish	
TLR	**Ligand**	**Source**	**Ligand**	**Source**
TLR1	Lipopeptides[1]; GPI[2]; Pam3CSk4[3]	Bacteria, parasites	Unknown	
TLR2	Lipopeptide[1]; peptidoglycan[4]; Pam2CSk4[3]	Bacteria	Lipopeptides[1]; Pam_3CSk_4[3]	Bacteria
TLR3	dsRNA[5]; polyI:C[6]	Virus	dsRNA[5]; polyI:C[6]	Virus
TLR3a/b	dsRNA[5]	Virus	Unknown	
TLR4	LPS[7]	G- bacteria	-	
TLR4b.a/b	dsRNA[5]	Virus	Unknown	
TLR5M	Flagellin[8]	Bacteria	Flagellin[8]	Bacteria
TLR5b	-		Unknown	
TLR5S	-		Flagellin[8]	Bacteria
TLR7	ssRNA[5]; R848[9]	Virus	Unknown	
TLR7a/b	-		-	
TLR8	ssRNA[5]	Virus	Unknown	
TLR8a/b	-		-	
TLR8a1/a2	-		-	
TLR9	CpG sites[10]	Virus, bacteria	Unknown	
TLR11	Profilin[11]; flagellin[8]	Bacteria, parasites	-	
TLR14	-		Unknown	
TLR19	-		Unknown	
TLR20a	-		Unknown	
TLR21	-		Unknown	
TLR22	-		dsRNA[5]; polyI:C[6]	Virus
TLR22a/b	-		Unknown	
TLR23	-		Unknown	

Table 3. TLRs and their ligands in fish and mammals based on Palti 2011. [1]Lipopeptides are found in the cell walls of bacteria. [2]Glycosylphosphatidylinositol (GPI) structures are found in eukaryotes and in particular in trypanosomatid parasites. [3]Pam_3CSk_4 and Pam_2CSk_4 are synthetic bacterial lipopeptides. [4]Peptidoglycan (murein) is found in the cell wall of eubacteria. [5]Single stranded RNA (ssRNA) and double stranded RNA (dsRNA) are found in RNA viruses, which have their genetic material as RNA. [6]Polyinosinic:polycytidylic acid (PolyI:C) is structurally similar to dsRNA but is expressed in the membrane of B-cells, macrophages and dendritic cells. [7]Lipopolysaccharide (LPS) is found in the outer membrane of Gram negative bacteria. [8]Flagellin is a substituent of the bacterial flagellum and present in nearly all flagellated bacteria. [9]R848 is a synthetic antiviral compound with low molecular weight and an agonist for mammalian TLR7/8. [10]CpG sites are regions of DNA or RNA with high occurrences of phosphodiester-bound cytosine and guanine nucleotides. [11]Profilin is found in e.g. *Toxoplasma gondii* and human uropathogenic *Escherichia coli*.

3.2.3. Innate humoral factors

Several immunologically active factors can be found in blood and other body fluids and are referred to as 'humoral' factors. The most important of these are complement, MBL and other opsonins (which facilitate phagocytosis), antimicrobial peptides, proteolytic enzymes, acute phase proteins and cytokines. These parameters may be classified e.g. based on their effector functions related to disease.

Complement system

A key feature in innate immunology is the complement system, which stimulate cellular defense. The complement system is an enzyme cascade system consisting of several proteins, which are found circulating in the blood. There are three pathways able to activate the complement system: The classical pathway, the alternative pathway, and the lectin pathway. These pathways are activated/initiated by different molecules. The classical pathway is activated by C1 binding to either an antibody:antigen complex or directly onto the bacterial surface. The lectin pathway is initiated by the binding of a C-type lectin to a pathogen (Murphy *et al.* 2008), but they also have an opsonizing[14] effect, thus augmenting phagocytosis and also playing a central role in activation of adaptive immunity. One of the most well-known C-type lectins is MBL. In rainbow trout, the characterization of a potential lectin pathway is in progress (Kania *et al.* 2010). The alternative pathway is initiated by the spontaneous binding of complement component C3 to pathogen surfaces. All three pathways result in the formation of the protease C3 convertase, which binds to the pathogen surface and cleaves C3, leading to a cascade of events with further cleavage and activation. The cumulative effect is 1) attraction of phagocytes, macrophages and neutrophiles, through chemotaxis, 2) enhanced phagocytosis through opsonins attaching to antigens, 3) removal of immune-complexes and 4) lysis of cells and pathogens by the interaction of the terminal components forming a membrane-attack complex creating pores in the cell surface (Murphy *et al.* 2008).

The complement system of teleosts differs from its mammalian counterpart at several points, including the existence of several isoforms of key complement components, such as C3. It has been hypothesized that the diversity in structure and function allow the immediate recognition of a broader range of microorganisms without processing antigens. Compared to mammals, teleosts have a very wide temperature range for complement activation and alternative complement activation results in high titers of related proteins. The combination of these circumstances makes complement a very important defense system in fish, which has probably evolved due to constant skin contact with the environment, including potential pathogens (Boshra, Li & Sunyer 2006).

Cytokines

Cytokines are involved in regulation of inflammation and immune responses. They are small proteins released by body cells and affecting the behavior of cells. There are two main classes of cytokines,

[14] Opsonin: antibody or product of complement activation found in the blood, which lead pathogens to become more susceptible to the action of phagocytes.

one of which is produced by lymphocytes and often referred to as lymphokines or interleukins (abbreviated IL). Despite their name, interleukins are produced by many types of cells. The other class is chemokines, which stimulate migration and activation of responsive cells, typically phagocytes and lymphocytes (Murphy *et al.* 2008). Various cytokines have different functions in inflammation, either by being regulatory, pro- or anti-inflammatory. The cytokines examined using qPCR in the present thesis are described below.

IL-10

In mammals, the regulatory anti-inflammatory cytokine IL-10 plays a central role in regulation of the inflammatory response. IL-10 is primarily associated with macrophages, both as a target and source, although many other cell types have been shown to secrete the cytokine. The exact pathways for secretion are unidentified, although several cytokines, including IL-12, IL-6, TGF-β and IL-27, are known mediators (Cyktor & Turner 2011). Inflammation is a balance between pro- and anti-inflammatory cytokines, and regulation is necessary to reduce damage caused by an excessive immune response. The key function of IL-10 in mammals is to regulate the expression of other cytokines, such as the pro-inflammatory cytokines IL-1β and IL-6, mainly on a transcriptional level (Seppola, Larsen, Steiro, Robertsen & Jensen 2008). Taken as a whole, the innate response is inhibited by an up-regulation of IL-10.

Few functional studies have been carried out in fish and the role of IL-10 remains unverified (Seppola *et al.* 2008). However, several studies indicate a role in teleosts similar to that described in mammals. For example, an increase in expression of the gene encoding IL-10 was observed in rainbow trout 3 days post infection with *Yersinia ruckeri* and no pro-inflammatory cytokines were up-regulated thereafter (Raida & Buchmann 2008b). Also, stimulation with the inflammatory agents LPS, flagellin, zymosan and β-glucan resulted in an IL-10 up-regulation followed by a decreased expression of the inflammatory molecules IL-6 and COX-2 (Chettri, Raida, Holten-Andersen, Kania & Buchmann 2011).

IL-17

In humans, the family of regulatory cytokines known as IL-17, consists of six members, IL-A to IL-F. IL-17A (often referred to simply as IL-17) and IL-17F are closely related and both located in tandem on the same chromosome. In mammals, IL-17 plays an important role in the stimulation of pro-inflammatory gene expression, and promotion of cellular infiltration through pro-inflammatory cytokines and chemokines (Kono, Korenaga & Sakai 2011). IL-17A/F is produced by numerous cell types, mainly leukocytes and especially the T_h17[15] subtype, which consist of differentiated CD4 + T cells. Expression is up-regulated during infection, where they play an important part in the recruitment of neutrophils (Wang, Martin & Secombes 2010). IL-17C is induced selectively in the epithelium by multiple independent pathways and is also up-regulated as a response to inflammation (stimulation with IL-1β and TNF) as well as bacterial challenge. IL-17C contribute to the inflammatory response by expression of inflammatory cytokines, chemokines and antimicrobial peptides similar to IL-17A/F. IL-17C has been shown to have both protective and inflammation

[15] The T_h17 subset of T helper cells has recently been discovered and is believed to be involved in defense against pathogens in mucosal tissues by stimulating epithelial cells to produce antimicrobial compounds.

promoting abilities, hence playing an important role in the regulation of the epithelial immune response (Ramirez-Carrozzi *et al.* 2011).

Teleost homologues to IL-17 genes have been found in many teleost species, including rainbow trout, where IL-17c1 and IL-17c2 were identified in 2010. The teleost IL-17C genes have been shown to be major responders to lipopolysaccharides (LPS) (Kono *et al.* 2011; Wang *et al.* 2010), a cell wall component in Gram negative bacteria, which act as endotoxins[16] and are known to elicit an immune response. An acute response within 1 - 4 hours after exposure has been observed in several teleost species, including trout. Furthermore, expression of trout IL-17c1 and IL-17c2 in macrophage cell lines was also up-regulated in response to other inflammatory stimulants, such as IL-1β, INF-γ and poly I:C. Thus, it seems expression is regulated by a number of pathways in trout and that the two genes seem to be regulated by different pathways (Kono *et al.* 2011). In healthy rainbow trout (100 g), expression of IL-17c1 dominated in gills and skin, while expression of IL-17c2 predominated in spleen, head kidney and brain. Expression of both genes was higher in gills, skin and intestine and was significantly up-regulated after injection with *Y. ruckeri* (Wang *et al.* 2010). Mucosal surfaces are potential portals of entry for pathogens; the results therefore indicate a function in host defense.

TGF-β

The regulatory cytokine Transforming Growth Factor-β (TGF-β) has several inhibitory effects in mammals, among others influencing proliferation of B and T cells and suppressing neutrophils and macrophages. In Atlantic salmon infected with *Moritella viscosus*[17], an up-regulation was seen for TGF-β expression after the peak of pro-inflammatory cytokines. In mechanically damaged rainbow trout, TGF-β and the pro-inflammatory cytokines were co-expressed (Ingerslev, Lunder & Nielsen 2010). TGF-β also seems to have an immunosuppressive role in salmonids, which is linked to regeneration of damaged tissue.

IL-1β

IL-1β is a pro-inflammatory cytokine in mammals and is associated with adhesion, colonization and invasion of bacterial pathogens in rainbow trout (Komatsu *et al.* 2009). The cytokine is immediately expressed following disturbance and injury to the epithelium (Gonzalez *et al.* 2007). IL-1β has been shown to participate in the initiation of immune response in trout to *Y. ruckeri* (Wang *et al.* 2009; Raida & Buchmann 2009) and an up-regulation after natural infection of rainbow trout with *F. psychrophilum* has been observed in gills, spleen, liver and head kidney (Orieux *et al.* 2013).

IL-6

In mammals, the pleiotropic pro-inflammatory/anti-inflammatory cytokine IL-6 is linked to regulation of hematopoiesis, inflammation, immune responses and bone homeostasis. The effects include T- and B-cell growth and differentiation, production of acute phase proteins and fever. IL-6 has a double role as both a pro- and anti-inflammatory cytokine; IL-6 is up-regulated by IL-1β and TNF-α, which are inhibited by the produced IL-6. Furthermore, IL-17 is dependent on IL-6 and

[16] Endotoxin: Toxin present inside an intact bacterial cell and released upon destruction of the cell.
[17] *M. viscosa* is a Gram negative bacteria and cause of winter ulcer disease.

needed for differentiation of T-cells into the T_H17 subtype (Murphy *et al.* 2008). A recent study indicated that IL-6 is likely to have many of the same effects in rainbow trout (Costa, Maehr, Diaz-Rosales, Secombes & Wang 2011).

IL-4/13A

In mammals, IL-4 is a cytokine which activate CD4+ cells, so that these differentiate into T_h2 cells, also known as T helper cells. T_h2 cells are predominantly involved in the stimulation of antibody producing B cells (Murphy *et al.* 2008). Furthermore, IL-4 has an anti-inflammatory effect by inhibiting production of pro-inflammatory cytokines (e.g. IL-1β and IL-6). A study on zebrafish indicated that the function of IL-4 has been conserved from fish to mammals over the course of vertebrate evolutionary history, although class switching is not induced in teleosts. IgM is considered to be the most indigenous antibody in mammals and appears to be the main antibody isotype involved in humoral immunity in fish. It is proposed that the original role of IL-4 was to participate in the production of IgM (Zhu, Pan, Fang, Shao & Xiang 2012). IL-4 and IL-13 are functionally closely related cytokines, which are localized in tandem on the genome. Whether the teleost IL-4/13 genes are orthologous to either IL-4 or IL-13 is unknown (Ohtani, Hayashi, Hashimoto, Nakanishi & Dijkstra 2008).

Acute phase proteins

The acute phase response is induced by pro-inflammatory cytokines such as IL-1β, and acute phase proteins are found in the blood shortly after onset of an infection (Murphy *et al.* 2008). Several acute phase proteins exist and although they all contribute to clearance or control of pathogens, their mode of action vary. Transferrin removes iron from damaged tissue by chelation and activates macrophages, while also functioning as a growth inhibitor by making iron unavailable for bacteria, while hepcidin inhibits iron transport. Another acute phase protein is CRP, which is a PRR and an opsonin, and which activate the complement system via the classical pathway. MBL, precerebellin and complement factors, such as C3 and C5, are also acute phase proteins. Serum amyloid A (SAA) is described in more detail below.

Serum amyloid A

SAA is another acute phase protein, which responds rapidly and participate in the early stages of damage and inflammation by recruiting leukocytes (Kania & Chettri 2013); its presence is thus a good indicator of the acute phase response. SAA is mainly produced in the liver and can be found in the blood stream soon after the onset of infection. Natural infection with *F. psychrophilum* and stimulation with associated PAMPs both induced SAA expression in the liver and lymphoid tissues of rainbow trout (Villarroel, Zambrano, Amthauer & Concha 2009). A similar response has been seen to *Y. ruckeri*, where an up-regulation was observed in the spleen, intestines and liver of infected rainbow trout (Chettri *et al.* 2012; Evenhuis & Cleveland 2012; Raida & Buchmann 2009; Raida & Buchmann 2008a). A comparison between immersion exposure to *F. psychrophilum* and injection with *Y. ruckeri* indicated, that SAA is very responsive to injection challenge (Evenhuis & Cleveland 2012). Furthermore, regulation of SAA seems to be highly individual, with variations even in constitutive expression in healthy fish (MMAH II).

Other humoral factors

Interferons (IFN) are involved in anti-viral defenses. There are many type I IFNs with structural homology, which all bind to the type I IFN receptor on the cell-surface. There is only one type II IFN, which binds to the type II IFN receptor. IFN-α and IFN-β are type I interferons secreted by cells infected with virus and are involved in the innate immune response. IFN-γ is a type II interferon, which is secreted by activated T-cells and NK cells and involved in immune regulation and inflammatory responses. Interferons also act as a growth inhibitor in viral infections by inducing expression of antiviral proteins.

Protease inhibitors are used in the defense against pathogens which secrete proteolytic enzymes. Lytic enzymes, like lysozyme, can work both alone and in cascades and are important in the defense against bacteria. Lysozyme is bactericidal and causes lysis and is mostly connected with Gram positive bacteria, although Gram negatives may also be lysed. Lysozyme also functions as an opsonin activating the complement system as well as phagocytes. Agglutins and precipitins, like C-type lectins, interact with carbohydrates, which lead to opsonization, phagocytosis and complement activation; MBL, which has been mentioned previously, is the most thoroughly studied C-type lectin. Although antibody is a feature belonging to adaptive immunity, natural antibodies are of an innate character. They are produced without specific antigen stimulation (not as a response to an antigen) and rearrangement of genes, and are found in the majority of vertebrates. They are often specific for highly conserved epitopes from intracellular structures, rather than from cell surfaces. The natural antibodies of rainbow trout have been shown to participate in the defense against both viral and bacterial pathogens (Magnadottir 2010).

3.3. Inflammation

Acute inflammation is a localized protective response in the tissue to injury or infection and is mainly initiated by macrophages and dendritic cells resident in the tissue. PAMPs are recognized by the PRRs on these cells and result in the release of inflammatory mediators. Most early inflammatory mediators increase movement of plasma and leukocytes into the tissue, leading to the development of the five hallmarks of inflammation: Redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and dysfunction of the organs involved (*functio laesa*). The general purpose of the inflammatory response is to destroy and remove pathogens, confine the area and reduce the spread of pathogens, stimulate and increase the immune response and incite wound healing (Murphy *et al.* 2008).

Neutrophils are the first leukocytes to be attracted to the site of inflammation by chemotaxis. Furthermore, the endothelial cell walls are activated by e.g. TNF-α and express cell-adhesion molecules, which along with the slower blood flow allow leukocyte migration into the tissue (Murphy *et al.* 2008). Neutrophils phagocytose bacteria, dead cells and cellular remains, before dying and being expelled from the body as pus. Secondly, the phagocytotic monocytes arrive, differentiating into macrophages or dendritic cells depending on cytokines in the environment. Macrophages are able to present antigens to the B and T cells, linking the response to adaptive

immunity. Furthermore, eosinophils are important contributors in combating parasites and the removal of fibrin[18]. Basophils are similar to mast cells and have cytoplasmic granules containing inflammatory mediators (e.g. histamine, bradykinin), which are released upon activation (Murphy *et al.* 2008).

The pro-inflammatory cytokines secreted by the phagocytes after detecting tissue damage or microbial products via surface receptors, include IL-6, TNF-α, IL-1β, CXCL8[19] and IL-12 and have several important functions: The non-immunological role of promoting tissue repair, to deliver effector molecules and cells to the infected area in order to boost pathogen killing and to induce local blood clotting to limit the spread of infection to the blood stream. Complement activation also mediates inflammation through the cleavage of C3, C4 and C5, which produce the anaphylatoxins C3a, C4a and C5a, which attract leukocytes. A protein fraction consisting mainly of C3a, C4a and C5a from rainbow trout blood has been shown to enhance phagocytosis by head-kidney leukocytes three- to four fold, which is not seen in mammals. Furthermore, the fraction also displayed chemo-attractant abilities (Boshra *et al.* 2006).

It is generally accepted that the level of inflammation is a balance between the pro-inflammatory and the anti-inflammatory cytokines. The anti-inflammatory cytokines include IL-10 and TGF-β, and while they seem to play a similar role in salmonids, their precise functions are still somewhat unclear.

3.4. Adaptive immune response

If a local immune response is not sufficient to control an infection, the pathogen will be transferred to central immune organs and the processing of antigens will initiate the adaptive immune response. The adaptive immune system consists of several components, including immunoglobulins (antibodies) and T cell receptors (TcR), T cell markers (CD3, CD4, CD8) as well as MHC I and II molecules. The functions of these components are described in more details in the following sections.

3.4.1. Antibodies

The recognition of a specific antigen and adaption of the immune response to it is unique for adaptive immunity. As previously mentioned, fish contain the RAG gene family and both rearranging T cell receptor and immunoglobulin genes, which allow a highly versatile response against antigens (Lieschke & Trede 2009). Acquired immunity is realized primarily through the antibodies and these may: 1) Neutralize toxins, 2) facilitate phagocytosis by opsonization or 3) active the classical complement pathway forming a protein complex on the pathogen surface resulting in direct destruction or phagocytosis (Murphy *et al.* 2008).

[18] Fibrin: Protein involved in blood clotting.
[19] CXCL8: Also known as IL-8.

In mammals, B cells generally need to be activated by helper T cells to differentiate into antibody producing plasma cells[20]. It takes 4-5 days after pathogen recognition by the innate immune system, until clonal expansion and differentiation of lymphocytes into effector cells is completed. This is called the primary antibody response and it has three main purposes: To clear infection, to temporarily prevent re-infection and to form immunological memory. Almost all of the plasma cells made during the primary response die within a few months, but a few activated antigen-specific B and T cells persist long after infection. These long-lived memory B and T cells allow for a faster and more effective secondary response. Firstly, they are more easily activated. Secondly, B-cells are more specific due to affinity maturation and isotype switching. Finally, they are more abundant than the naïve specific cells. As a result, re-infections are usually cleared before causing disease – even for pathogens with high mortality rates (Murphy *et al.* 2008; Parham & Janeway 2009).
In jawed fish and ectothermic vertebrates in general, the overall performance of antibodies is slower and weaker compared to mammals (Rauta, Nayak & Das 2012). Antibodies produced by fish are of a lower affinity and diversity compared to mammals, since only a low level of heterogeneity is observed in the antibodies produced to a single hapten (Randelli, Buonocore & Scapigliati 2008). Furthermore, there is no class switching and the typical secondary response in mammals, which is dependent on memory cells, is not seen in fish. Secondary antibody response is e.g. amplified to a lesser degree and the response time for antibody production for salmonids is at least 4-6 weeks and dependent on temperature (Magnadottir 2006; Ellis 2001; Olsen et al. 2011).

Immunoglobulins are only found in jawed vertebrates and cartilaginous and teleost fishes are the first phylogenetic group in which they appear. Five different classes are present in mammals, IgM, IgA, IgD, IgE and IgG, while only three have been found in teleosts: IgM, IgD and IgT/IgZ. Although IgM is the predominant antibody produced by teleosts, levels in the blood are subject to variation between species (Uchida et al. 2000). Teleost IgM typically exists in tetrameric form and, although it has many similarities to its mammalian counterpart, there are also differences. So called 'redox forms' are formed after treatment with denaturing agents, when the non-covalent bonds of the tetramer structure are interrupted, causing dissociation into a variety of H:L chain combinations. In addition, IgM can be secreted as both a monomer and dimer structure. This plasticity may compensate for some of the lacking isotype diversity by enhancing the epitope binding ability of IgM (Watts, Munday & Burke 2001).
Although IgA plays a significant role in mammals, where it is associated with mucosal surfaces, the class has not been found in teleosts. Naturally, the immune response of skin and gills in fish is especially important, since both are in constant direct contact with the surroundings. Consequently, IgM was believed to act both systemically and in the mucosal surfaces until the discovery of IgT in rainbow trout and IgZ in zebrafish (Hansen, Landis & Phillips 2005; Danilova, Bussmann, Jekosch & Steiner 2005; Zhang *et al.* 2010). This new class was subsequently found in many teleost species and has later been proven to exist in both gut mucus and serum, although to a lesser extent in the

[20] T-cell independent antigens exist. They can directly stimulate the production of antibody without T helper cells through two mechanisms; either co-stimulation of the B cell is provided by a TLR or multiple repeating epitopes on the antigen allowing activation through cross-linking.

latter. Furthermore, the same study proved the existence of a new B cell line only expressing IgT on its surface, and that this line constituted the majority of B cells in the gut-associated lymphoid tissue in rainbow trout (Zhang *et al.* 2010) and in gill epithelia (Olsen *et al.* 2011).
Finally, homologue forms of IgD have been found in all jawed vertebrates, except for Aves (birds). IgD appears on the surface of mature and naïve B cells in mammals, but its function is still unknown. Although more research is needed to examine distribution and function of IgD, a secretory form has also recently been revealed in all immune tissues of rainbow trout albeit at a lower level than IgM (Ramirez-Gomez *et al.* 2012).

3.4.2. Adaptive cellular response

The co-receptors CD4 and CD8 facilitate binding between an antigen presenting cell and the T cell receptor, also called T_CR. The T_CR consists of a heterodimer comprised of a highly variable alpha chain and the invariant beta (β) chain, which recognizes and binds peptide:MHC ligands. Signaling to the cell goes through the associated CD3 complex (Murphy *et al.* 2008). Monitoring the regulation of the TcR-β-chain gives an indirect measure of the presence of T-cells in a sample. Several subsets with distinct functions of each T cell exist. T cells have been recognized in teleosts since the 70s, although knowledge about them is still limited (Randelli *et al.* 2008).

CD8+ T cells carry the co-receptor protein CD8 and recognize intracellularly-derived peptides. The antigens are assembled with their presenting MHC class I molecules in the endoplasmatic reticulum and afterwards displayed on the cell surface. These T cells differentiate into cytotoxic T cells in mammals. Regulation of CD8 has previously been shown to be repressed in rainbow trout naturally infected with *F. psychrophilum* (Orieux *et al.* 2013), while being up-regulated in the spleen of injection-challenged fish (Overturf & LaPatra 2006).

CD4+ T cells carry the co-receptor protein CD4 and recognize peptides bound to MHC class II molecules, which are derived extracellularly. They differentiate into T_h1 and T_h2 effector cells, which respectively activate macrophages and stimulate antibody production through B cell proliferation. MHC II has previously been shown to be repressed in rainbow trout naturally infected with *F. psychrophilum* (Orieux *et al.* 2013).

Foxp3 is a transcription factor specific to T cells and plays an essential role in the development of regulatory T cells (T_{reg}), as well as in the regulation of the immune response during inflammation in mammals. In rainbow trout, two paralogue genes have been identified: FoxP3a and FoxP3b. Analysis of structure and function has revealed differential modulation of the two genes and hence the possibility for novel functions (Wang *et al.* 2010). A study in Atlantic salmon indicated a similar role, although the existence of T_{reg}-like T cells has yet to be proven in this species (Zhang, Chi, Niu, Bogwald & Dalmo 2011). A steady increase of Foxp3 transcripts was found in Atlantic salmon infected with the bacterial pathogen *Aeromonas salmonicida*, with the highest expression 48 hours after intraperitoneal challenge (Zhang *et al.* 2011).

Conclusion

Life in an aquatic environment entails permanent contact with the surroundings through the mucosal surfaces, such as the gills, which also serve as site of entry for many pathogens. The immune system of fish is in many ways comparable but not identical to what is seen in mammals. One of the fundamental differences is the function of the head kidney in fish, which is the site of hematopoiesis and equivalent to the bone marrow in mammals. The immune system is classically divided into two parts: The innate and the adaptive. The innate components are less specific and fast-acting, whereas adaptive immunity is acquired and takes time to develop and protect against reoccurring infections with the same pathogen. Although immunity is not fully developed in younger life stages of rainbow trout, regulation of immune-relevant genes takes place and high constitutive expression of e.g. IgT may protect against pathogens. Phagocytes are important in the first line of defense and presentation of antigens after pathogen-engulfment is necessary to initiate the adaptive response, which e.g. leads to the production of antibodies. The efficiency of antibodies in fish is generally lower compared to mammals; they take longer to develop, and the secondary antibody response is less evident.

4. Immune response in rainbow trout to *F. psychrophilum*

Previous studies regarding the immune response in rainbow trout after exposure to *F. psychrophilum* have relied on either naturally infected or injection-challenged rainbow trout. Although injection-based challenge with *F. psychrophilum* is a standardized approach, it is suboptimal for the purpose of investigating the immune response, since mucosal surfaces are bypassed. The use of naturally infected fish entails several complications which may confound interpretation of data; adaptive immune responses take time to develop from initial contact with a pathogen, and the fish may be affected by additional unknown pathogens and other factors such as various forms of stress. The size of the fish in the studies varied from 1 to 100 g, but the majority of fish weighed from 10 – 50 g. Results of previous studies regarding gene expression related to infection with *F. psychrophilum* are summarized below. In studies where pathogen load was correlated with changes in gene expression, the strength of a correlation is expressed as Pearson's *r*. The *r*-value denotes the strength of a linear dependence between two variables. The strength of a correlation is considered to be very weak: 0.00 - 0.19, weak: 0.20 - 0.39, modest: 0.40 - 0.69, strong: 0.70 - 0.89 and very strong: 0.90 - 1.00.

Villarroel *et al.* (2008)

One study focused solely on the expression of SAA in various tissues of naturally infected rainbow trout and found an up-regulation of the SAA gene in the intestine, skin, liver and gills. Furthermore, the study indicated that SAA is likely to be involved in local rather than systemic defense, since it was not found in the plasma of diseased fish.

Orieux *et al.* (2013)

The study used naturally infected rainbow trout (1 - 100 g) and samples originated from 7 different farms and were collected over 6 months. All fish potentially affected by cross-infections were discarded[21] and no uninfected controls were used for qPCR. The fish were divided into two groups based on a visual assessment: "Normally appearing" (n = 37) and "diseased" (n = 24). The visually "diseased" fish were identified by necrotic tissue, lack of appetite and a swollen abdomen. The bacterial titer in each fish was determined using 16S rRNA qPCR; some individuals had bacterial levels below the limit of quantification in all tissues. The "diseased" group was subdivided into two groups: Fish treated with antibiotics (n = 8) and untreated fish (n = 16). The "normally appearing" group included fish in which *F. psychrophilum* could be detected with qPCR in varying degree; some were below the lower limit for quantification, while others were free of the bacterium in some or all tested tissues. The group thus potentially included fish which were 1) healthy, 2) developing or overcoming RTFS and 3) survivors of recent infections (and possibly healthy carriers). An array of genes related to stress and adaptive immunity were examined and the results are shown in Table 4. No correlations were found between pathogen load and regulation of gene expression.

[21] The selection process was not described in the paper.

	Gills		Liver		Spleen		Kidney	
	T	UT	T	UT	T	UT	T	UT
mt-a	5.5	4.7	4.3	-	6.6	2.7	5.0	-
sod1	-	-	-	-	-	-	-	-
IL-1β	12.7	4.6	35.2	13.0	7.6	3.5	27.2	14.6
IFN-γ	-	-	-	-	-	-	-	-
TGF-β	-	-	-	-	-	-	-	-
CD8	-	-	-	-	0.1	0.3	-	-
CD4	-	-	-	-	-	-	-	-
MCH II	-	-	-	0.3	0.4	2.3	-	-
IgM	-	-	-	11.8	-	-	-	-
IgT	-	-	-	-	0.03	-	0.04	-

Table 4. Orieux *et al.* (2013). Significant regulations (p =0.05) of gene expression found in naturally infected fish (Orieux *et al.* 2013). The "normally appearing" group was used as control and regulation of gene expression was compared to the visually "diseased" fish. The diseased fish were subdivided into two groups: Untreated (UT) and treated (T) with antibiotics.

Overturf & LaPatra (2006)

The study used 20 fish (14 g) subcutaneously injected with the virulent strain CSF-259-93 in three doses: Low, medium and high (CFU were not stated, although plate counts on TYES were carried out at the time of injection). Five fish were sampled 1 and 5 days post-infection. No significant changes in regulation were identified after 1 day and except for regulation of the C3 gene in the liver, the only significant changes were found in the group injected with a medium dose of *F. psychrophilum*. The results are shown in Table 5. A considerable individual variation was also noted by the authors.

	Liver	Kidney	Spleen
CD8	-	2.24	3.69
C3	2.4[1]	31.24	6.05
MX-1	-	12.19[2]	9.92
IL-8	-	3.78	6.24

Table 5. Overturf & LaPatra (2006). Significant regulations (p =0.05) of gene expression 5 days post-infection (Overturf & LaPatra 2006). No significant changes were found 1 day post-infection. Unless stated otherwise, the results are from the group receiving a medium dose of *F. psychrophilum*. [1]Regulation of C3 in liver was only significant in the high dose group. [2]MX-1 was also significantly up-regulated (0.14-fold) in the low dose group.

Evenhuis & Cleveland (2012)

The study used juvenile rainbow trout (40 - 50 g) and 8 samples were taken 3 and 10 days post intraperitoneal injection with 1.4 x 10^7 CFU *F. psychrophilum*. Regulation of gene expression and correlation with bacterial load in the intestine is shown in Table 6. A large increase in SAA transcription was observed after both 3 and 10 days. The results were compared to regulation after *Y. ruckeri* bath-challenge and it was concluded that SAA expression seems very responsive to injection challenge.

	Correlations (r)		Regulation (fold)	
	3 d	10 d	3 d	10 d
SAA	0.39	0.71	162	277
IL8	-	0.84	6	5
IFN-γ	-	0.79	-	6.5
TNF-α	-	0.57	2	3
TGF-β	-	0.54	-	-
TLR5	-	-	-	-
IgM	-	0.70	3.4	12.1
IgT	-	-	-	-
TCR-β	-	-	-	-

Table 6. Evenhuis & Cleveland (2012). Significant regulation (p =0.05) of gene expression in the intestine after intraperitoneal injection (Evenhuis & Cleveland 2012).

Bruun, Raida & Dalsgaard (2009)

A small unpublished study examined the immediate innate immune response in the liver of 1-3 g rainbow trout vaccinated with either formalin-killed *F. psychrophilum* or purified outer membrane vesicles (OMV). Samples were taken after 4 h, 12 h and 24 h and changes in the regulation of the following genes was investigated: IL-1β, IL-6, TNF-α, TLR-5, SAA, trout C polysaccharide binding protein, pentraxin (CRP/SAP-like), precerebellin, transferrin, hepcidin, C3, C5 and factor B. A low constitutive expression was observed for all genes in the control group. Only regulation of TLR-5 differed significantly after 4 and 12 h post vaccination with OMV. Since *F. psychrophilum* does not have a flagella, it was concluded that it receptor activation was induced in another and yet unknown manner.

MMAH II (2013)

The immune response in the head kidney of rainbow trout weighing 1.2 ± 0.5 g after immersion in *F. psychrophilum* and/or H_2O_2 was examined. Samples for examination of gene expression were taken 4, 48, 125 and 192 hours post-infection from four experimental groups: 1) Untreated controls, 2) fish treated with H_2O_2, 2) *F. psychrophilum* and 3) H_2O_2 + *F. psychrophilum*. The regulation of an array

of genes primarily related to adaptive immunity was examined and changes in expression were correlated with pathogen load: FoxP3a, FoxP3b, IL-17c1, IL-17c2, IL-10, IL-6, IgT, IgM, SAA, CD8, CD4, MHC I, MHC II, TcR- β, IL-4 and IL-1β. The statistically significant results are summarized in Table 7.

	4 h				**48 h**	
	H_2O_2	***F.p.***	**H_2O_2 + *F.p.***	**H_2O_2**	***F.p.***	**H_2O_2 + *F.p.***
IL-10				↓*	↓**	↓**
IL-1β		↑*	↑*			
IL-17c1						↓↓↓*
IgT			↓↓**			
MHC I		↑*	↑**			
	125 h			**192 h**		
	H_2O_2	***F.p.***	**H_2O_2 + F.p.**	**H_2O_2**	***F.p.***	**H_2O_2 + F.p.**
IL-10				↓↓***	↓↓**	↓↓*
IL-1β				↓**	↓**	
IL-17c1				↓↓↓***	↓↓***	↓↓**
FoxP3a					↓↓**	
FoxP3b					↓↓***	↓**
IgT		↓↓**				
MHC II		↓**	↓**			
IL-4				↓*	↓↓***	
TcR-β					↓**	

Table 7. MMAH II (2013). Significant changes in regulation of gene expression in the head kidney. The fish were exposed to either H_2O_2, *F. psychrophilum* (*F.p.*) or H_2O_2 and *F. psychrophilum* (H_2O_2 + *F.p.*). 3-5 fold changes are designated by ↑/↓, 5-10 fold by ↑↑/↓↓ and 10-13 fold changes by ↑↑↑/↓↓↓. Red arrows signify down-regulations, while green arrows signify up-regulations. Significance levels are denoted with asterisks (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Four hours after immersion in *F. psychrophilum*, the genus specific 16S rRNA was already present in the head kidney. In the H_2O_2 + *F. psychrophilum* group, the amount of genus specific 16S rRNA increased steadily, although the difference was not significant until 192 h post infection. The high initial and subsequent decrease of 16S rRNA in the *F. psychrophilum* group may be an indication of a response to the infection, which leads to greater survival, possibly through antigen processing and presentation. Bacteria are phagocytosed in mature rainbow trout, by which the production of reactive oxygen species is triggered and the pathogen is killed. Virulent strains of *F. psychrophilum* have been shown to have a higher rate of survival inside macrophages in mature rainbow trout (LaFrentz,

LaPatra, Jones & Cain 2003). Thus, the increasing amount of 16S rRNA in the H_2O_2 + *F. psychrophilum* group may reflect the host's inability to clear the infection.

All significant correlations ($p < 0.01$ or $p < 0.05$) in the study were strong or very strong, except for FoxP3a (0.68). For the *F. psychrophilum* group, positive correlations were found between pathogen load and regulation of FoxP3a, IgT, IgM, SAA, CD4 and MHC I, while IL-17c1 first positively correlated and later negatively correlated. For the H_2O_2 + *F. psychrophilum* group, a positive correlation was observed for regulation of SAA, IgM and IL-10. A pro-inflammatory response was observed in both infected groups. A delay in correlation was observed for IgM, which may indicate a lag in production of antibodies early in infection. However, no statistically significant difference in *F. psychrophilum*-specific antibody levels was seen 50 days post-challenge measured by ELISA. If there was a difference in the initial period, the fish may have had time to compensate, resulting in comparable levels of specific antibodies at the later stage. The absence of a clear pattern in the immune response could be partly explained by a high individual variation of gene regulation, which has been encountered before (Overturf & LaPatra 2006).

MMAH III (2013)

The immune response in the gills after immersion in *F. psychrophilum* and/or H_2O_2 was examined using the same setup as for the head kidney (MMAH II), and the statistically significant results are summarized in Table 8.

	4 h			**48 h**		
	H_2O_2	***F.p.***	**H_2O_2 + *F.p.***	**H_2O_2**	***F.p.***	**H_2O_2 + *F.p.***
IL-10						↓**
IL-1β	↑**	↑**	↑**			
SAA						
IL-17c1			↑**	↓↓***		↓↓***
IgT		↓↓*				
	125 h			**192 h**		
	H_2O_2	***F.p.***	**H_2O_2 + *F.p.***	**H_2O_2**	***F.p.***	**H_2O_2 + *F.p.***
IL-1β		↑↑*				
SAA			↑*			↑*
IL-17c1	↓↓***					
IgT	↓↓↓***					
CD8	↑↑*				↑*	

Table 8. MMAH III (2013). Significant changes in regulation of gene expression in the gills. The fish were exposed to either H_2O_2, *F. psychrophilum* (*F.p.*) or H_2O_2 and *F. psychrophilum* (H_2O_2 + *F.p.*). 3-5 fold changes are designated by

↑/↓, 5-10 fold by ↑↑/↓↓ and 10-13 fold changes by ↑↑↑/↓↓↓. Red arrows signify down-regulations, while green arrows signify up-regulations. Significance levels are denoted with asterisks (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

The relative pathogen load was also determined for the two infected groups by quantification of 16S rRNA, but no significant differences were found at any time point. Pathogen load was correlated with changes in gene expression, and a negative correlation was found for IL-17c1, MHC I and MHC II in the *F. psychrophilum* group, while IL-4/13A and IgM correlated positively in the H_2O_2 + *F. psychrophilum* group.

It has been demonstrated that IL-17c1 is mainly expressed in gills and skin, and even though the effect in fish is still unknown, the gene has been shown to be significantly up-regulated after injection-challenge with *Y. ruckeri* (Wang *et al.* 2010). The up-regulation of CD8, although not correlated with pathogen load, indicated an adaptive response to *F. psychrophilum*. CD8 positive T cells recognize antigens presented on MHC I molecules and differentiate into cytotoxic T-cells, which are related to intracellular infections. Whether an up-regulation of the CD8 gene was delayed beyond the time-frame of the study or absent in the H_2O_2 + *F. psychrophilum* group is unknown.

Conclusion

It is difficult to compare the results from the various studies in an overall interpretation due to differences regarding method of infection. Furthermore, some of the fold-regulations taken into consideration in the interpretation of the data in previous studies are low. In the work carried out during the present thesis, only changes $\geq$ 3-fold and $p \geq 0.05$ were considered significant. Furthermore, additional information may be gained by correlating pathogen load and gene expression in the individual fish. A pro-inflammatory response is indicated in both gills and head kidney after infection with *F. psychrophilum*, but the adaptive response was vague and highly influenced by pre-treatment with H_2O_2. In head kidneys from the *F. psychrophilum* group, a positive correlation between pathogen load and regulation of the IgT, IgM, CD4 and MHC I genes was observed, which was altered to a delayed correlation with IgM by pre-treatment with H_2O_2. The delay in regulation of IgM was not reflected by the antibody production 50 days post-exposure. IL-10 also positively correlated in the H_2O_2 pre-treated group, which could be taken as an indication of a suppressive effect elicited by *F. psychrophilum*. In the gills, a negative correlation was seen for MHC I and MHC II, and pre-treatment with H_2O_2 resulted in only a positive correlation with regulation of IgM. Hence, no distinct evidence for either a T_h1 or T_h2 response was observed in either infected group, but exposure to H_2O_2 before infection with *F. psychrophilum* altered the immune response.

5. Aquaculture

The term aquaculture refers to the breeding, rearing and harvesting of aquatic species in any type of watery environment. Rainbow trout (*Oncorhynchus mykiss*) is the most prominent species in Danish aquaculture and constituted 92 % of the production from 2003 to 2011 (Table 9), amounting to over 85 % of the total value (Ministeriet for Fødevarer, Landbrug og Fiskeri 2006). The world's food fish production from aquaculture has increased 12-fold from 1980 to 2010, with production reaching an all-time high in 2010 with 60.000 million tons of food fish produced, amounting to a value of US $ 119 billion (FAO 2012). The yearly production of rainbow trout in Danish aquacultures is around 40.000 tons and the governmental strategy is to further increase the production (Ministeriet for Fødevarer, Landbrug og Fiskeri 2013).

Production of rainbow trout in Danish aquaculture									
Year	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Tons	35.287	40.454	37.065	34.912	39.080	38.526	36.906	36.518	37.531

Table 9. Tons rainbow trout produced in Danish aquaculture. In 2011, the total value amounted to approx. 680 million DKK of a total value of approx. 800 million DKK for all aquaculture production (Ministeriet for Fødevarer, Landbrug og Fiskeri 2013).

5.1. Rainbow trout

Rainbow trout were initially described and named by Johann Julius Walbaum in 1792. They are carnivorous fish native to the Pacific Ocean in Asia and North America. Coloration varies with habitat and farmed specimens are typically grey with a blue- or yellow-green shade and a pink streak along the side. The dorsal side of the body is covered with small black spots, while the ventral side is white or silvery. The average adult size is 60 cm, although much larger individuals have been reported. In nature, rainbow trout are found in cold creeks, rivers and lakes. In freshwater environments, rainbow trout feed on various invertebrates and smaller fish, while they mainly prey on fish and cephalopods in seawater. They thrive well under hatchery conditions and have therefore been introduced in many countries for sport or farming purposes (Froese & Pauly 2013).

5.2. Farming, health and management

The optimal rearing temperature for eggs and fish up to a size of about 50 g is 7 °C, at which temperature hatching takes place after 300-350 day degrees (≈ 45 days) post fertilization. After about 120 day degrees (14-20 days) post-hatching, the yolk sack is depleted and the fry will begin to feed. The fry stage (Figure 6) lasts for 500 day degrees (≈ 10 weeks), at which point the fish weigh 5 g and are called fingerlings (Jokumsen & Svendsen 2010). Fry and fingerlings are usually produced at one farm and subsequently sold to production facilities elsewhere for on-growing until a saleable size is reached.

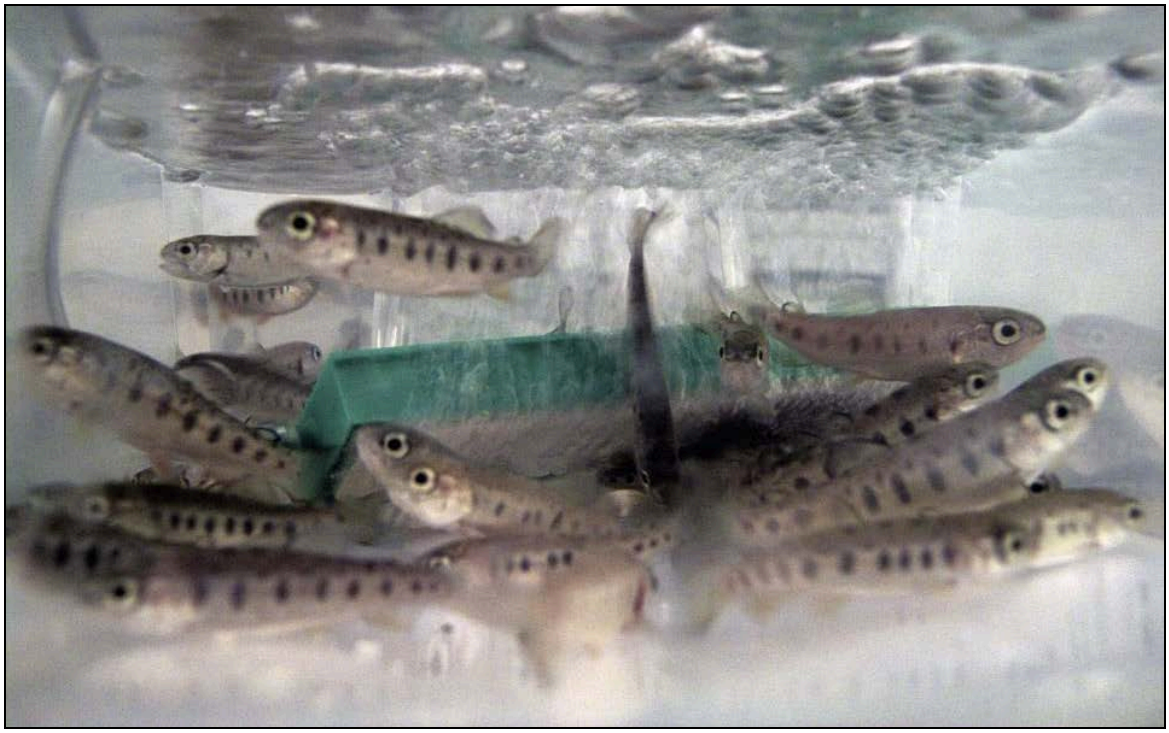


Figure 6. Rainbow trout fry weighing approximately 1 g.

Large-scale animal production in general is more profitable than smaller-scale operations, but also more exposed to disease outbreaks. Keeping the fish healthy is the key to securing animal welfare and high productivity, and to avoid the spread of disease. Both national and EU regulations exist for the transportation of fish of all stages of development to hinder the spread of disease (Jokumsen & Svendsen 2010). Changes in the environment or unfavorable conditions may stress the fish and make them more predisposed for disease as a result of physical damage, stress or both.

Antibiotics and non-medicine compounds are commonly used in trout farming. While antibiotics require a prescription, the application of non-medicine therapeutic substances is less restricted. These agents (Table 10) are used in fish farming for various purposes, e.g. to improve water quality, as a disinfection or against ectoparasites. Eggs and milk, for example, are stripped from the brood stock and disinfected carefully after fertilization. To avoid fungus attacks, a fungicide like formaldehyde is applied with regular intervals (Jokumsen & Svendsen 2010).

Substance	Unit	2001	2002	2003	2004	2005
Lime	(tons)	1.243	1.100	1.130	779	964
Formaline*	(L)	108.843	134.751	151.284	65.571	40.314
Copper sulfate	(kg)	7.294	8.772	7.747	3.398	2.090
Chloramine-T	(kg)	7.352	8.769	7.147	4.905	2.494
Hydrogen peroxide	(L)	4.178	7.210	5.271	7.561	1.961
Sodium carbonate	(kg)	11.696	23.703	3.598	9.503	2.333
Sodium chloride	(kg)	400	67.100	41.200	31.525	63.881
Benzalkonium chloride	(kg)	0	0	10	2	15

Table 10. Estimates of non-medicine therapeutical chemicals used on Danish fish farms. Based on Jokumsen & Svendsen 2010 and By- og landskabstyrelsen (Danish Agency for Spatial and Environmental Planning) 2009 .

5.3. Hydrogen peroxide and the gills

Bath-treatments with formalin products are routinely used against pathogens in aquaculture, but this practice is to be phased out in Denmark. By using and encouraging substitution with other substances, the aim is to phase out formalin due to human health considerations. Hydrogen peroxide (H_2O_2) and peracetic acid (CH_3CO_3H) products have proven useful as alternatives to formalin for many treatments; in Denmark, they have been used for over ten years against skin parasites, bacterial gill infection, mold on eggs and for stripped female brood fish (Pedersen 2010; Sortkjær *et al.* 2000).

Hydrogen peroxide decomposes into water and oxygen ($H_2O_2 + H_2O_2 \rightarrow H_2O + O_2$), making it a very environmentally friendly alternative to formalin. Moreover, H_2O_2 has a fast turnover rate. H_2O_2 is primarily degraded by microbial activity and the decomposition rate is positively correlated with initial concentration, organic matter and water temperature. To maintain a constant concentration over a longer period of time H_2O_2 must be added continuously (Pedersen 2010). The concentration can be monitored to assess the decomposition rate, e.g. by using commercially available peroxide test sticks (Sigma-Aldrich).

The extent of harmful effects due to H_2O_2 treatment of fish depends on several factors, including applied concentration, exposure time and frequency of treatment (Arndt & Wagner 1997); longer exposure to higher concentrations result in mortality, although the mortality rates are subject to variation between studies. LC_{50} at 15 °C for rainbow trout fry weighing 0.26 g has previously been determined to be 514 mg, 322 mg and 207 mg L^{-1}, when treated for 30, 60 and 120 minutes, which was similar to the results for fry weighing 7.3 g (Arndt & Wagner 1997). The size of the fish is also essential, since larger fish are more at risk to damage from the treatment (Rach, Schreier, Howe & Redman 1997). Finally, treatment with H_2O_2 is generally discouraged at temperatures above 14 - 15°C (Sortkjær *et al.* 2000; Arndt & Wagner 1997; Kiemer & Black 1997). Recommended treatment dosages at 13 °C compared to treatment at 11 °C and below are reportedly 2/3 lower (Sortkjær *et al.*

2000). Treatment of rainbow trout fry at 14°C with H_2O_2 did not, however, result in significant mortality in the present work (MMAH I).
Mortality due to H_2O_2 exposure occurs within the first days after treatment, predominantly during or within hours of exposure (Tort *et al.* 2002; Gaikowski, Rach & Ramsay 1999). The various studies are difficult to compare directly. Thus, performance of pilot studies on a smaller number of fish under the relevant conditions is recommended before treatment of an entire population (Rach *et al.* 1997; Gaikowski *et al.* 1999).

The deleterious effect of H_2O_2 on fish tissue has previously been demonstrated to be confined to the gills (Tort *et al.* 2002). In rainbow trout, damage manifested as increased epithelial cell granularity, edemas, lamellar fusion, necrosis and swelling and lifting of the gill epithelium (Derksen, Ostland & Ferguson 1999; MMAH III), which is shown in Figure 7.

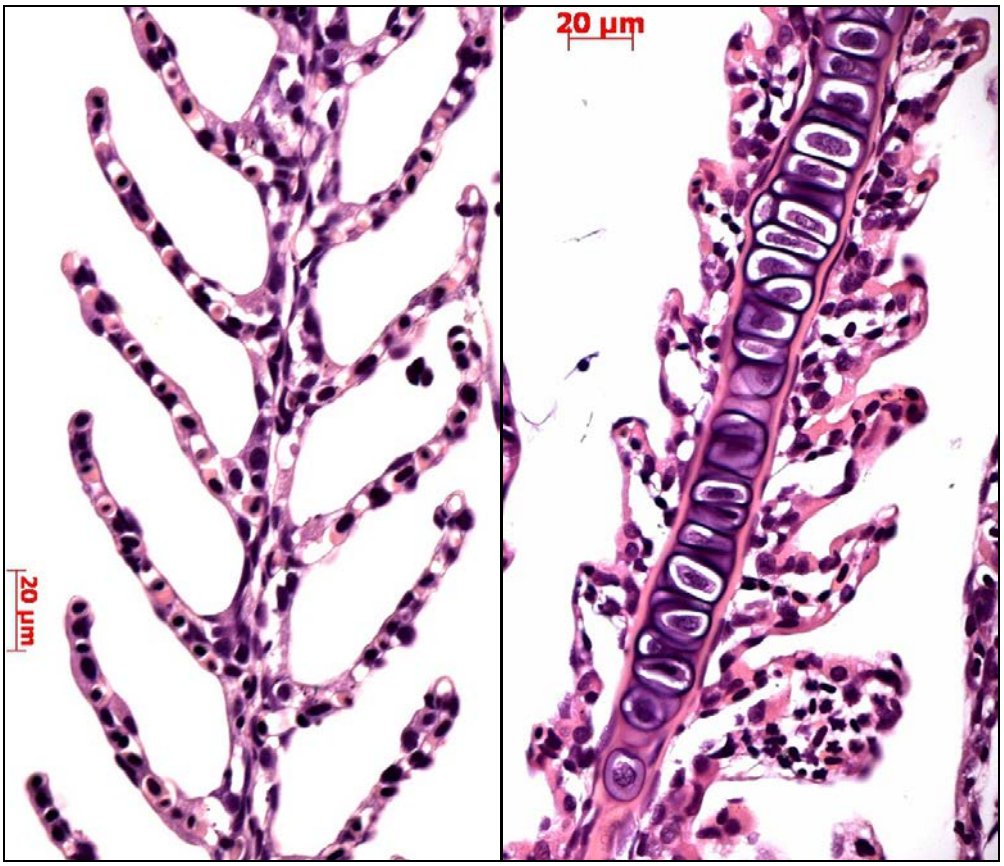


Figure 7. The effect of H_2O_2 on the gills. The structure of a normal gill (left) compared to the disrupted secondary lamella on the gills 4 hours post exposure to 150 mg L^{-1} H_2O_2 for 60 minutes (right) (MMAH III).

In the present study, an escape response was observed after exposure to concentrations between 50 and 300 mg L^{-1} H_2O_2, which was within the previously recommended safe treatment range. Furthermore, both acute mortality during exposure (2 - 3.5 %) and mortality during the experimental period (4 – 5 %) were observed. The experimental setup was not designed to determine the prolonged effect on mortality after a single H_2O_2 treatment, but the results indicate the possibility of excess mortality, especially if pathogens are present.

Gill diseases and injury

Gill diseases are very common in salmonids, although they are inadequately understood, and treatments are consequently suboptimal. Bacterial gill disease (BGD) e.g. commonly affects salmonids bred in aquacultures and thus subjected to intensive conditions. The causative agent of BGD is *Flavobacterium branchiophilum*, forming characteristic aggregates in the gills. When environmental conditions deteriorate, the pathogen is able to attach and invade its host and lead to high mortality rates. Despite of the economic consequences of these outbreaks, knowledge regarding the disease is still limited (Good, Thorburn & Stevenson 2008). Nodular gill disease (NGD) is often mistaken clinically for BGD. NGD is associated with amoebae infestations and is often clinically invisible, until trout are exposed to a chemical treatment, at which point even low doses of well-tolerated chemicals can cause massive mortality. Deaths following a period of heavy feeding may be observed in such cases ((Ferguson 2006; Tubbs, Wybourne & Lumsden 2010)).

There are three main types of initial interaction between a pathogen and the gill tissue: 1) Colonization of the gills, 2) trapping of systemically distributed pathogens within the gills' phagocytically- and endocytotically active cells and 3) interaction between a waterborne toxin and the lamellar epithelium. Regardless of the type of initiation, secondary tissue responses seem to be of a more general character regarding both disease and injury. This suggests that damage may have a defensive function for the host. Repair of the gill tissue is often fast, and although few studies have focused on the time frame of epithelial repairs, it is expected to match the time of transit from epithelium to the tip of lamella. However, repair following complete destruction of lamellae due to H_2O_2 exposure begins within several weeks (Ferguson 2006).

5.4. Stress

Animals in large-scale productions are exposed to many potential stressors (Table 9) and aquatic animals are even more vulnerable, since they are in constant contact with their surrounding environment. Furthermore, fish are poikilothermic, i.e. their body temperature follows the ambient temperature. If possible, rainbow trout seek out warmer or colder water to regulate their body temperature (Gräns, Rosengren, Niklasson & Axelsson 2012). Large amounts of blood run through the gills and contact with cold water thus leads to a rapid loss of heat, while metabolic activity increases the body temperature. Changes in temperature may therefore also cause stress (Harper & Wolf 2009).

Chemical	Physical	Biological	Procedural
Poor water quality	Temperature	Crowding	Capture
Contaminants	Light	Microorganisms	Handling
Diet composition	Sounds	Macroorganisms	Shipping
Metabolic waste	Dissolved gasses	Malnutrition	
Disease treatment	Hypoxia	Disturbance	

Table 9. Examples of possible stressors in fish farming (Harper & Wolf 2009; Francis-Floyd 1990).

There are many issues connected with evaluating stress. Firstly, the definition of the concept is controversial, inexact and the term is applied inconsistently. Stress can be defined as the total response of a given organism to a stressor, leading to a state where homeostasis is threatened, i.e. the organism is no longer able to maintain stable internal properties, causing the normal physiological state to be disrupted. The organism will try to respond to re-establish homeostasis. Secondly, the sampling procedure by itself may cause a stress reaction. Thirdly, stress responses are very complex. Lastly, morphological changes caused by stress can be very hard to differentiate from tissue damage and various compensatory adaptations (Harper & Wolf 2009).

Stress is an alarm reaction, causing the animal to be in a state of fight or flight. Energy is metabolized to escape an immediate danger, which is advantageous and outweighs the temporary deleterious side-effects, e.g. on the immune system. When maintained for shorter periods of time, it allows adaptation and response to urgent danger. In a farming setting however, the animals have difficulty avoiding the stressors, and the beneficial aspect of the response are absent, while the animals suffer from the harmful side-effects of prolonged or severe exposure to stress (Pickering 1992).

The responses following stress can be divided into three phases. The primary response is a generalized neuroendocrine response, in which the catecholamines epinephrine and norepinephrine are released along with the glucocorticoid cortisol (Harper & Wolf 2009; Ashley 2007). The release of these hormones triggers the secondary phase, in which physiological responses give rise to physiological and metabolic alterations (Schreck 2010). In this state of alarm, blood sugar is increased by hormone-induced metabolizing of stored sugars, resulting in the release of an energy reserve. The mineral metabolism is changed and osmoregulation disrupted. This encompasses over-hydration for freshwater fish and dehydration for saltwater fish. Respiration and blood pressure are also increased. Stored red blood cells are released and the inflammatory response is suppressed by released hormones, including cortisol (Francis-Floyd 1990). Hence, measurements of cortisol are complemented by measuring glucose and lactate levels in plasma (Ashley 2007), although both cortisol and glucose are highly variable. Furthermore, cortisol may be useful only in acute stress experiments (Martinéz-Porchas, Martinéz-Córdova & Ramos-Enriquez 2009). The long-term consequences of prolonged, repeated or unavoidable stress can result in progression to the tertiary phase, which include both direct and indirect maladaptive effects; such as growth reductions,

repressed reproduction, weakened immune response and decreased disease resistance (Ashley 2007). If the energy reserves are used up, the animal dies from exhaustion or disease due to increased susceptibility (Francis-Floyd 1990).

Exposure to chemicals in the water is unquestionably stressful. The following histopathological changes may reflect tissue damage caused by toxic properties of the chemical or a stress reaction. Whether the changes can be called stress depends on the definition used, since it may be argued that a condition is only a result of stress, if the changes can be directly linked to the release of stress hormones (Harper & Wolf 2009). The gills are delicate structures and constantly exposed to the immediate environment and are thus frequent targets of physical and chemical stressors. Some species of fish respond to low oxygen levels with adaptive morphological changes to the gills, while most respond less efficiently. With the current level of knowledge, it seems that almost any stressor may induce gill lesions as a part of a stress response (Harper & Wolf 2009).

5.4.1. Stress in rainbow trout

It has previously been reported that rainbow trout respond to harmful stimuli by elevating the concentration of cortisol in their plasma. A single treatment with H_2O_2 has previously been shown to have a short-term stress effect on Atlantic salmon, resulting in elevated concentrations of glucose and cortisol in the plasma (Bowers, Speare & Burka 2002).
Both confinement and treatment with formalin, which is used as treatment against e.g. *Ichthyophthirius multifiliis*, resulted in elevated cortisol levels (Jørgensen & Buchmann 2007). I.p. injection with hydrocortisone in rainbow trout had an effect on the molecular level of gene expression in the gills; most of the examined immune-related genes were down-regulated. Regulation of IgT seemed more sensitive to hydrocortisone compared to IgM, and both were down-regulated. The signaling molecules IL-1β, IL-8, IL-22 and IFN-γ were also down-regulated, as was the CD8, CD4 and MHC II genes, which are related to adaptive immunity (Olsen *et al.* 2011).
A previous study examined the effect of acute and chronic stress on the phagocytic capacity of macrophages in spleen and head kidney. Acute stress was induced in one of two ways: I.p. injection under anesthesia or noise vibration for 1 hour followed by 2 hours of confinement. Chronically stressed fish were injected daily with either saline or dexamethasone[22]. Furthermore, cells were subjected to either cortisol or an adrenergic antagonist[23]. The amount of yeast cells engulfed per macrophage (n = 200) was determined. Acute stress reduced the number of phagocytic events with ≈30 - 50 %. When stress became chronic, the effect on phagocytosis was less pronounced, although plasma cortisol levels were comparable to levels after acute stress. Incubation with cortisol did not affect phagocytic activity. The results showed that acute stress can significantly depress the number of phagocytically active macrophages and that the effect is at least partly attributable to the effect of catecholamines (Narnaware, Baker & Tomlinson 1994).

[22] Dexamethasone is a synthetic anti-inflammatory and immunosuppressant glucocorticoid steroid.
[23] Adrenergic antagonists have an effect similar to epinephrine/adrenaline and suppress cortisol.

In another study, rainbow trout macrophages[24] were stimulated with cortisol, which resulted in a direct inhibition of IL-6 and IL-8, while suppressing the up-regulation of various pro-inflammatory cytokines. Similarly, cortisol caused a 33 % reduction in IL-8 expression following exposure to LPS. Furthermore, IL-10 was up-regulated in the cells in the absence of cortisol (Castro, Zou, Secombes & Martin 2011).

Conclusion

Rainbow trout is the most common fish species in Danish aquaculture. Large-scale production of animals may be more economically profitable but also more vulnerable to disease outbreaks, and both antibiotics and non-medicine chemicals are commonly used. Besides the use of chemicals, physical, biological and procedural conditions may also stress or damage the fish and make them vulnerable to pathogens. The increased mortality observed after pre-treatment with H_2O_2 before challenge with *F. psychrophilum* may have been caused by physical disruption of the outer barriers of the fish or mediated through stress. It has been determined that exposure to H_2O_2 induces short-term stress in Atlantic salmon and sea bass (Bowers *et al.* 2002; Roque, Yildiz, Carazo & Duncan 2010). The morphological changes after exposure to H_2O_2 observed in the present study correlate well with previous studies, though fused lamellae were rare. Exposure to either H_2O_2 or *F. psychrophilum* both resulted in damages to the gills and the tissue was in recovery 192 hours post-exposure. However, when exposed to both, edemas and epithelial lifting was still evident at this time-point. The presence of *F. psychrophilum* 16S rRNA in the gills was demonstrated using qPCR, although no bacteria were observed with either FISH or on the H&E stained tissue sections. Thus, the gills as a portal of entry in RTFS cannot be excluded.

[24] RTS-11: rainbow trout phagocytic monocyte/macrophage-like cell line.

6. Methodological considerations

The following section describes the various methods applied in the present thesis. Materials and methods are included for techniques which are not described in the accompanying articles.

6.1. Cultivation and confirmation of *F. psychrophilum* by PCR

The procedure for cultivation of *F. psychrophilum* has been described in MMAH I. In brief, samples were taken from brain, kidney and spleen. Brain and kidney samples were streaked onto TYES and blood agar plates[25], while spleen samples were placed in TYES broth (110 rpm). Since *F. psychrophilum* is fastidious and exhibits slow growth, TYES broth and plates were incubated for 7 days at 15 °C. Yellow colonies growing only on TYES and any growth in TYES broth were further examined using species-specific PCR. Initially, a pure culture was obtained by streaking, and after 5-7 days of growth a single colony was suspended in 50 µL distilled water and boiled for 5 min to lyse the bacterial cells. Afterwards, the lysed bacteria were placed on ice and PCR was carried out (Wiklund, Madsen, Bruun & Dalsgaard 2000).

PCR is a highly sensitive technique used for amplification of specific DNA sequences and is widely applied in many fields, ranging over medicine, diagnostics and forensics. In PCR, complementary primers (forward and reverse) specific to the DNA sequence of interest are mixed with DNA polymerase. Together, these components enable the exponential synthesizes of copies in the presence of nucleotides during repeated amplification of the template DNA. Overall, a PCR cycle consists of three steps. 1) Denaturation: The DNA is separated into two single strands by heating. 2) Annealing: The primers anneal to their target sequence. 3) Extension: The polymerase synthesizes the DNA-strand from the primers, resulting in two double-stranded DNA molecules.

The method was reliable when applied in the present work. Visual assessment of the yellow colonies was done in all cases, but PCR was also used to verify the identity of bacteria re-isolated from experimentally challenged fish. The use of PCR is especially important regarding samples from less controlled environments than experimental infections, since the possibility for misidentification with other yellow bacteria is greater.

6.2. Reverse-transcription PCR (RT-PCR)

RT-PCR, also called qPCR, was used to assess up- and down-regulations of selected genes at the transcriptional level in MMAH II and MMAH III. RNA was converted to copy DNA (cDNA) by using reverse transcriptase. Under optimal conditions, the quantity of the desired DNA sequence will double for each cycle. Two types of probes were used: TaqMan and SYBR Green. TaqMan probes are more specific and the basic principle is the cleaving of dual-labeled probes, resulting in the emission of increasing fluorescence, which allows quantitative detection during the exponential

[25] Nutrient rich mediums with blood may be used for differential diagnostics, since *F. psychrophilum* will not grow on them.

phase of DNA replication. TaqMan probes are double-labeled with a fluorophore in the 5'-end and a quencher in the 3'-end. While the fluorophore and quencher are in close proximity to one another, fluorescence signals are inhibited. TaqMan probes bind to a region between the forward and reverse primer. When the Taq polymerase extends the primer during PCR, the probe is degraded in the process. The fluorophore is released and emits fluorescence in the absence of the quencher. SYBR Green binds to all double-stranded DNA, including non-specific PCR products such as primer-dimers, and does not emit a fluorescent signal unless it is bound. During the denaturation step of the PCR cycle, the SYBR Green will be released into the solution and bind again during extension. A melting curve analysis is carried out after RT-PCR is completed by raising the temperature slowly. This leads to the dissociation of DNA and a drop in fluorescence, which can be visualized. Thus, it is possible to see whether all samples have the same melting temperature or if several peaks occur. A primer-dimer will be seen as a peak at a lower temperature, since the DNA strand is shorter.

The RT-PCR method for detection of *F. psychrophilum* (Orieux, Bourdineaud, Douet, Daniel & Le Hénaff 2011) was not optimal, since unspecific replication was observed in the uninfected controls. Furthermore, the sensitivity under the conditions in the present study was not as high as in the original paper. The various assays for immune-relevant genes worked satisfactorily, although changes in regulation of the chosen genes did not fully elucidate the immune response after infection with *F. psychrophilum*. The use of microarrays may offer more insight, especially when neither a T_h1 nor T_h2 response was evident, since the expression level of many genes are examined simultaneously.

6.3. Fluorescent in situ hybridization (FISH)

Fluorescent probes are also used in FISH, where they bind to specific DNA sequences and are visualized through fluorescent microscopy. FISH was used to detect the location of *F. psychrophilum* cells in tissue sections from experimentally infected rainbow trout. The sample is hybridized with a fluorescence-tagged probe, which is complementary to a short fraction of the target DNA sequence. The sample is incubated with the probe to allow binding. Afterwards, the excess probe is washed away and the sample can be examined using fluorescent microscopy. By using various colors, a sample can be incubated with several probes. This way several fractions may be distinguished in the same sample; a general eubacterial probe may e.g. be tagged with green fluorescence and a bacteria of interest may be tagged with red. Multiple staining with a general bacterial probe and a specific probe also allows positive signals from the specific probe to be verified and distinguished from auto-fluorescence in tissue.

In the present study, the heads of 2 fish from each replicate (4 per group in total) were fixed in 10 % neutral buffered formalin for 24 h (room temperature) and stored in 70 % ethanol. The secondary gill arch was removed from the head and embedded in paraffin separately. The rest of the head was cross-sectioned, so that brain, nasal cavity and eyes were visible. Samples and sampling for FISH was identical with the procedure described for H&E-stained tissue.

The tissue samples were embedded in paraffin blocks and FISH was carried out according to a previously published and validated method (Liu *et al.* 2001). In brief, two species-specific primers (PSY1 and PSY2) targeting the 16S rRNA genes of *F. psychrophilum* were used (Toyama *et al.* 1994). The primers were labeled with Cy3 (TAG Copenhagen) and diluted to a concentration of 1 ng μl^{-1} in-situ hybridization buffer (50 ml 1M Tris (pH 7,2), 90 ml 5 M NaCl, 5 ml 10 % SDS and H_2O to a total volume of 500 ml). Furthermore, the sections were double-stained with a previously published eubacterial probe ((Amann *et al.* 1990)) for validation of signals. After incubating the slides for 24 hours at 45 °C, they were washed three times with hybridization buffer and three times with in-situ wash buffer (50 ml 1 M Tris (pH 7.2), 90 ml 5 M NaCl and H_2O) to a total volume of 500 ml). Afterwards, the slides were washes in MilliQ water for 1 minute, before air drying in the incubator at 45 °C. The slides were stored at 4 °C in darkness until microscopic examination took place using a Zeiss Axioimager M1 epifluorescence microscope equipped with a 120-W HBO lamp. Filter set 38 was used for detection of fluorescein, filter set 43 for detection of Cy3 and filter set 24 for simultaneous detection of both green and red fluorescence. Micrographs were taken with an AxioCam MRm v. 3 FireWire monochrome camera using AxioVision software, v. 4.5 (Carl Zeiss).

The presence of *F. psychrophilum* 16S rRNA was demonstrated in the gills by qPCR, suggesting that the negative results obtained using FISH warrant verification. It is more difficult to consolidate a negative result than a positive, and the gills as a potential portal of entry cannot be excluded on the background of the present results. The FISH method must be validated despite that it has been published in order to reach a more definite conclusion regarding the gills as a potential portal of entry for *F. psychrophilum*.

6.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a sensitive technique, which is typically applied in the field of diagnostics. In the present thesis, ELISA was used for detection of antibody in blood samples (Figure 11). Plates were coated with *F. psychrophilum* antigen and were incubated with samples containing varying amounts of antibody. Once the antibody was allowed to bind to the antigens, a mouse anti-salmonid antibody was applied to bind to the antibodies from the blood samples. Subsequently, an enzyme-labeled rabbit anti-mouse antibody was added, which was bound to the mouse antibody. Finally, the substrate for the enzyme was added, which resulted in a change in color depending on the antibody levels in the blood sample.

The ELISA methods used in the present study were reliable. The plates were coated with sonicated bacteria, which is ideal in an experimental setting, since the plates may be coated with the same bacteria used for the challenge. This is, however, a potential source of error when examining the antibody levels of naturally infected animals, if there are differences in antigens on the bacteria.

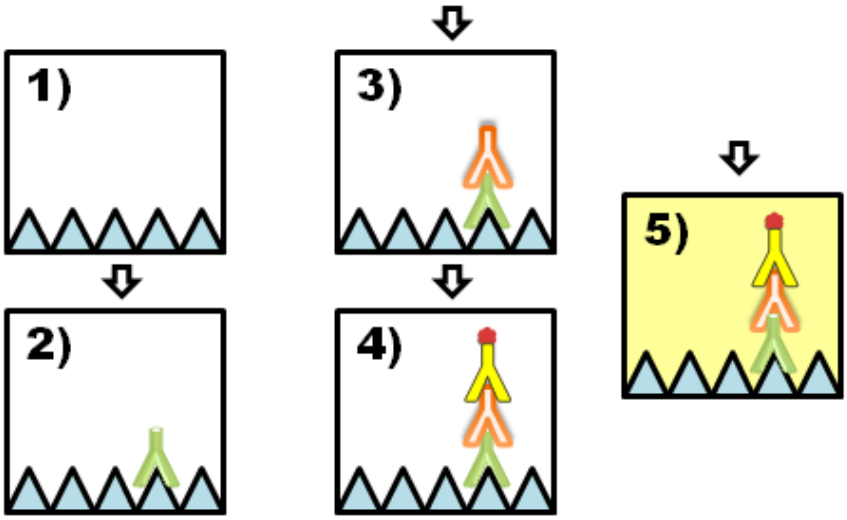


Figure 11. Indirect ELISA used for detection of specific antibodies. 1) The wells were coated with antigen (sonicated bacterial pellet). 2) Plasma from blood samples was added to each well and specific antibodies were bound to the antigens. 3) Mouse anti-salmonid antibody was added and bound to the specific antibodies in the serum. 4) HRP-labeled rabbit anti-mouse antibody was added, binding to the mouse anti-salmon antibody. 5) TMB substrate was added and converted by the HRP enzyme, resulting in a color reaction.

7. Concluding remarks and perspectives for future research

Despite great focus on research both nationally and internationally, many aspects of RTFS are not yet fully understood. Followingly, *F. psychrophilum* continues to be a major cause of mortality in farmed rainbow trout. Part of the scope of the present thesis was to elucidate the immune response in rainbow trout fry with RTFS in order to improve prevention of the disease. It has previously been speculated that RTFS outbreaks could be linked to external factors. Various non-medicine compounds such as formalin and H_2O_2 are used routinely in aquaculture against pathogens. Alone, H_2O_2 elicited an immune response in both gills and head kidney, and exposure to H_2O_2 prior to infection with *F. psychrophilum* was demonstrated to increase mortality and alter the immune response. Furthermore, increased mortality from a single treatment with H_2O_2 was shown. The potential impact on mortality of using these compounds, both alone and in combination with various pathogens, should be investigated further. Since the immune response to *F. psychrophilum* was scarce, the subject could be elucidated further by combining exposure to H_2O_2 with a pathogen eliciting a more thoroughly described immune response.

Adherence of *F. psychrophilum* to the gills could not be proven and the gills remain a potential portal of entry in RTFS. Although other studies have not been able to demonstrate an impact of H_2O_2 on other mucosal surfaces than the gills, these should be investigated further regarding both potential adhesion of *F. psychrophilum* and potential damage caused by H_2O_2. Furthermore, the first phase of contact between pathogen and host may be examined using e.g. optical projection tomography to reveal the portal(s) of entry.

The immune response to *F. psychrophilum* does not result in either a T_h1 or T_h2 response, and a potential suppression of the immune system was indicated. The regulation of other genes, also related to innate immunity, may be of interest. The spleen would also be an interesting organ to study in order to elucidate the response further. The use of microarray for screening of gene regulation after infection should be considered, since many genes can be examined simultaneously. The immune response in larger rainbow trout or less RTFS-susceptible rainbow trout families may also be compared to the response in rainbow trout to elucidate potential differences in the immune response, which may lead to greater survival.

Finally, the connection between virulence and growth stage should be investigated further. A previous study demonstrated an increased mortality after immersion-challenge with *F. psychrophilum* in the logarithmic phase of growth, and preliminary results indicate that adherence to the host may be affected. This would both require studies regarding possible changes in the bacterium during growth and challenge to assess the effect on mortality.

Acknowledgements

I wish to thank my supervisor, Associate Professor Inger Dalsgaard, for introducing me to the world of fish diseases in general and *Flavobacterium psychrophilum* in particular. We have had some very rewarding discussions over the past years and I have learned a lot from you.

I would also like to thank my co-supervisors, Professor Kurt Buchman and Associate Professor Per Walter Kania from the Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology at the University of Copenhagen (LIFE). You have been a great help and all your input has been highly appreciated.

Senior Researcher Lone Madsen has also been a great help, especially regarding the challenge experiments.

The laboratory technicians in Section for Bacteriology, Pathology and Parasitology at the National Veterinary Institute also deserve thanks, especially Lene Gertman, who has provided indispensable help when needed, and Annie Ravn Pedersen for helping out with tissue sections.

Professor Henrik Spliid from Section for Statistics, Informatics and Mathematical Modeling, DTU, and Senior Researcher Claes Enøe from Section for Epidemiology, DTU, also have my appreciation for discussing results and statistics with me.

Thanks to my fellow PhD-students at DTU: Gunilla Veslemøy Bech-Nielsen, Beata Renata Jonach, Rói Hammershaimb Christiansen, Anders Mellerup and Simona Bartkova for sharing inspiring, annoying and fun times! Thanks to all the PhD-students, post-docs and what-not at LIFE as well!

A big thanks to the rest of you wonderful people I've met through the past three years, both in Denmark and abroad.

Last, but definitely not least, a big thanks to my family, particularly Magdalena, Jens-Otto, Mazi, Laika and Lasse.

This work was funded by Technical University of Denmark (DTU) and the Danish Fish Immunology Research Centre and Network (DAFINET - http://www.dafinet.dk) with support from the Danish Council of Strategic Research.

References

Amann R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. & Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* **56,** 1919-1925.

Amend D.F. (1981) Potency testing of fish vaccines. *Developments in Biological Standardization* **49,** 447–454

Aoki M., Kondo, M., Kawai, K. & Oshima, S. (2005) Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Diseases of aquatic organisms* **67,** 73-79.

Arndt R.E. & Wagner, E.J. (1997) The toxicity of hydrogen peroxide to rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clark*i fry and fingerlings. *Journal of the World Aquaculture Society* **28,** 150-157.

Ashley P.J. (2007) Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science* **104,** 199-235.

Avendaño-Herrera R., Magariños, B., Irgang, R. & Toranzo, A.E. (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). *Aquaculture* **257,** 104-110.

Bernardet J.F. & Grimont, P.A.D. (1989) Deoxyribonucleic Acid Relatedness and Phenotypic Characterization of *Flexibacter columnaris sp.* nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986. *International Journal of Systematic Bacteriology* **39,** 346-354.

Bernardet J.F. & Kerouault, B. (1989) Phenotypic and genomic studies of "*Cytophaga psychrophila*" isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Applied and Environmental Microbiology* **55,** 1796-1800.

Bernardet J., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996) Cutting a Gordian Knot: Emended Classification and Description of the Genus *Flavobacterium*, Emended Description of the Family *Flavobacteriaceae*, and Proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *International Journal of Systematic and Evolutionary Microbiology* **46,** 128.

Borg A.F. (1960) Studies on myxobacteria associated with diseases in salmonid fishes. *American Association for the Advancement of Science* **8,** 1-85.

Boshra H., Li, J. & Sunyer, J.O. (2006) Recent advances on the complement system of teleost fish. *Fish and Shellfish Immunology* **20,** 239-262.

Bowers J.M., Speare, D.J. & Burka, J.F. (2002) The effects of hydrogen peroxide on the stress response of Atlantic Salmon (*Salmo salar*). *Journal of veterinary pharmacology and therapeutics* **25,** 311-313.

Bruno D.W. (1992) *Cytophaga psychrophila* (='*Flexibacter psychrophilus*') (Borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the UK. *Bulletin of the European Association of Fish Pathologists* **12,** 215-216.

Bruun M.S., Raida, M.K. & Dalsgaard, I. (2009) Bath vaccination with *Flavobacterium psychrophilum* outer membrane vesicles: Up regulation of toll-like receptor in liver tissue. In: *14th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Czech Republic, Prague, pp. 200.

Buchmann K., Bresciani, J. & Jappe, C. (2004) Effects of formalin treatment on epithelial structure and mucous cell densities in rainbow trout, *Oncorhynchus mykiss* (Walbaum), skin. *Journal of Fish Diseases* **27,** 99-104.

Buchmann K., Nielsen, M.E. & Nielsen, C.V. (2003) Immune responses against *Yersinia ruckeri* have no effect on colonization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), by *Gyrodactylus derjavini* (Mikailov, 1975). *Journal of Fish Diseases* **26,** 183-186.

Busch S., Dalsgaard, I. & Buchmann, K. (2003) Concomitant exposure of rainbow trout fry to *Gyrodactylus derjavini* and *Flavobacterium psychrophilum*: effects on infection and mortality of host. *Veterinary parasitology* **117,** 117-122.

By- og landskabstyrelsen [Danish Agency for Spatial and Environmental Planning] (2009) *Punktkilderapport 2007*, By- og landskabstyrelsen, Miljøministeriet, Copenhagen, Denmark, from www.naturstyrelsen.dk.

Cabello F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology* **8,** 1137-1144.

Cahill M.M. (1990) Bacterial Flora of Fishes: A Review. *Microbial ecology* **19,** 21-41.

Castro R., Zou, J., Secombes, C.J. & Martin, S.A. (2011) Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line. *Fish & shellfish immunology* **30,** 215-223.

Chettri J.K., Raida, M.K., Kania, P.W. & Buchmann, K. (2012) Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. *Developmental and comparative immunology* **36,** 463-474.

Chettri J.K., Raida, M.K., Holten-Andersen, L., Kania, P.W. & Buchmann, K. (2011) PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*). *Developmental and comparative immunology* **35,** 476-482.

Costa M.M., Maehr, T., Diaz-Rosales, P., Secombes, C.J. & Wang, T. (2011) Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: Effects on macrophage growth and antimicrobial peptide gene expression. *Molecular immunology* **48,** 1903-1916.

Cyktor J.C. & Turner, J. (2011) Interleukin-10 and Immunity against Prokaryotic and Eukaryotic Intracellular Pathogens. *Infection and immunity* **79,** 2964-2973.

Dalsgaard I. (1993) Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. *Annual Review of Fish Diseases* **3,** 127-144.

Dalsgaard I. & Madsen, L. (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *Journal of Fish Diseases* **23,** 199-209.

Danilova N., Bussmann, J., Jekosch, K. & Steiner, L.A. (2005) The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nature immunology* **6,** 295-302.

Declercq A.M., Van den Broeck, W., Haesebrouck, F., Bossier, P., Chiers, K., Dewulf, J., Cornelissen, M. & Decostere, A. (2013) Interactions of highly and low virulent *Flavobacterium columnare* strains with the gills of carp (*Cyprinus carpio*). *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 126.

Decostere A., D'Haese, E., Lammens, M., Nelis, H. & Haesebrouck, F. (2001) *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. *Journal of Fish Diseases* **24,** 481-487.

Decostere A., Lammens, M. & Haesebrouck, F. (2000) Difficulties in experimental infection studies with *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) using immersion, oral and anal challenges. *Research in veterinary science* **69,** 165-169.

Derksen J.A., Ostland, V.E. & Ferguson, H.W. (1999) Effects of hydrogen peroxide on clearance of formalin-killed *Flavobacterium branchiophilum* from the gills of trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **22,** 59.

Dumetz F., Duchaud, E., LaPatra, S.E., Le Marrec, C., Claverol, S., Urdaci, M.C. & Le Henaff, M. (2006) A protective immune response is generated in rainbow trout by an OmpH-like surface antigen (P18) of *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology* **72,** 4845-4852.

Ellis A.E. (2001) Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and comparative immunology* **25,** 827-839.

Ellis A.E. (1989) The immunology of teleosts. In: *Fish Pathology* (ed. by R.J. Roberts), pp. 135-52. Bailliére Tindall, London.

Evenhuis J.P. & Cleveland, B.M. (2012) Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. *Veterinary immunology and immunopathology* **146,** 8-17.

Fange R. (1986) Lymphoid organs in sturgeons (Acipenseridae). *Veterinary immunology and immunopathology* **12,** 153-162.

FAO (2000) *The State of World Fisheries and Aquaculture 2000*, Food And Agriculture Organization Of The United Nations, Rome, Italy.

FAO (2012) *The State of World Fisheries and Aquaculture - 2012*. Food And Agriculture Organization Of The United Nations, Rome, Italy.

Ferguson H.W. (2006) *Systemic pathology of fish, 2nd edition* (ed. by H.W. Ferguson). Scotian Press, London, UK.

Francis-Floyd, R. (1990) *Stress - Its Role in Fish Disease*, CIR919, Fisheries and Aquatic Sciences Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Froese R. & Pauly, D. (2013) *Oncorhynchus mykiss* (Walbaum, 1792). Retrieved September 19, 2013, from www.fishbase.org.

Gaikowski M.P., Rach, J.J. & Ramsay, R.T. (1999) Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. *Aquaculture* **178,** 191-207.

Garcia C., Pozet, F. & Michel, C. (2000) Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. *Diseases of aquatic organisms* **42,** 191-197.

Gonzalez S.F., Huising, M.O., Stakauskas, R., Forlenza, M., Lidy Verburg-van Kemenade, B.M., Buchmann, K., Nielsen, M.E. & Wiegertjes, G.F. (2007) Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory responses to injury mimicking infection with ectoparasites. *Developmental and comparative immunology* **31,** 244-254.

Good C.M., Thorburn, M.A. & Stevenson, R.M. (2008) Factors associated with the incidence of bacterial gill disease in salmonid lots reared in Ontario, Canada government hatcheries. *Preventive veterinary medicine* **83,** 297-307.

Gräns A., Rosengren, M., Niklasson, L. & Axelsson, M. (2012) Behavioural fever boosts the inflammatory response in rainbow trout *Oncorhynchus mykiss*. *Journal of fish biology* **81,** 1111-1117.

Guo P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, A. & Cooper, M.D. (2009) Dual nature of the adaptive immune system in lampreys. *Nature* **459,** 796-801.

Hadidi S., Glenney, G.W., Welch, T.J., Silverstein, J.T. & Wiens, G.D. (2008) Spleen Size Predicts Resistance of Rainbow Trout to *Flavobacterium psychrophilum* Challenge. *J Immunol* **180,** 4156-4165.

Hansen J.D., Landis, E.D. & Phillips, R.B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 6919-6924.

Harper C. & Wolf, J.C. (2009) Morphologic Effects of the Stress Response in Fish. *Ilar Journal* **50,** 387-396.

Högfors E., Pullinen, K.R., Madetoja, J. & Wiklund, T. (2008) Immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with a low molecular mass fraction isolated from *Flavobacterium psychrophilum*. *Journal of Fish Diseases* **31,** 899-911.

Högfors-Rönnholm E. & Wiklund, T. (2010) Phase variation in *Flavobacterium psychrophilum*: characterization of two distinct colony phenotypes. *Diseases of aquatic organisms* **90,** 43-53.

Holt R.A. (1987) *Cytophaga psychrophila,* the Causative Agent of Bacterial Cold-water Disease in Salmonid Fish.

Holt R.A., Rohovec J.S. & Fryer J.L. (1993) Bacterial coldwater disease. In: *Bacterial Diseases of Fish (ed. by V. Inglis, R.J. Roberts & N.R. Brombage)* pp. 3-23. Blackwell Scientific Publications, Oxford.

Hunnicutt D.W. & McBride, M.J. (2000) Cloning and characterization of the *Flavobacterium johnsoniae* gliding-motility genes gldB and gldC. *Journal of Bacteriology* **182,** 911-918.

Ingerslev H.C., Lunder, T. & Nielsen, M.E. (2010) Inflammatory and regenerative responses in salmonids following mechanical tissue damage and natural infection. *Fish and Shellfish Immunology* **29,** 440-450.

Izumi S. & Wakabayashi, H. (1999) Further study on serotyping of *Flavobacterium psychrophilum*. *Fish Pathology* **34,** 89-90.

Jayasuriya N.S., Herath, T.K., Bron, J.E., Adams, A., Mullins, J., McGurk, C. & Thompson, K.D. (2013) Gene expression profiles in Atlantic salmon gills after vaccination and challenge with *Aeromonas salmonicida* sub species *Salmonicida*. *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 94.

Jokumsen A. & Svendsen L.M. (2010) *Farming of freshwater rainbow trout in Denmark.* DTU Aqua report no. 219-2010, National Institute of Aquatic Resources, Technical University of Denmark, Charlottenlund.

Jooste P.J. & Hugo, C.J. (1999) The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. *International journal of food microbiology* **53,** 81-94.

Jørgensen T.R. & Buchmann, K. (2007) Stress response in rainbow trout during infection with *Ichthyophthirius multifiliis* and formalin bath treatment. *Acta Ichthyologica et Piscatoria* **37,** 25-28.

Kania P.W. & Chettri, J.K. (2013) Serum Amyloid A Gene expression and immunohistochemical localization in rainbow trout, *Oncorhynchus mykiss*, infected by *Yersinia ruckeri*. In: Dafinet Workshop and Ph.D. Course: Book of abstracts, Copenhagen, Denmark, pp. 27.

Kania P.W., Sorensen, R.R., Koch, C., Brandt, J., Kliem, A., Vitved, L., Hansen, S. & Skjodt, K. (2010) Evolutionary conservation of mannan-binding lectin (MBL) in bony fish: Identification, characterization and expression analysis of three bona fide collectin homologues of MBL in the rainbow trout (*Onchorhynchus mykiss*). *Fish and Shellfish Immunology* **29,** 910-920.

Kato G., Takano, T., Sakai, T., Matsuyama, T. & Nakayasu, C. (2013) *Vibrio Anguillarum* bacterin introduced through gill epithelium induces inflammatory responses in gills of Japanese flounder *Paralichthys Olivaceus*. *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 95.

Khimmakthong U., Deshmukh, S., Chettri, J.K., Bojesen, A.M., Kania, P.W., Dalsgaard, I. & Buchmann, K. (2013) Tissue specific uptake of inactivated and live *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*): Visualization by immunohistochemistry and in situ hybridization. *Microbial pathogenesis* **59-60,** 33-41.

Kiemer M.C.B. & Black, K.D. (1997) The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. *Aquaculture* **153,** 181-189.

Komatsu K., Tsutsui, S., Hino, K., Araki, K., Yoshiura, Y., Yamamoto, A., Nakamura, O. & Watanabe, T. (2009) Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. *Developmental and comparative immunology* **33,** 499-506.

Kondo M., Kawai, K., Kurohara, K. & Oshima, S. (2002) Adherence of *Flavobacterium psychrophilum* on the body surface of the ayu *Plecoglossus altivelis*. *Microbes and Infection* **4,** 279-283.

Kono T., Korenaga, H. & Sakai, M. (2011) Genomics of fish IL-17 ligand and receptors: A review. *Fish & shellfish immunology* **31,** 635-643.

Küchler A.M., Gjini, E., Peterson-Maduro, J., Cancilla, B., Wolburg, H. & Schulte-Merker, S. (2006) Development of the Zebrafish Lymphatic System Requires Vegfc Signaling. *Current Biology* **16,** 1244-1248.

LaFrentz B.R., LaPatra, S.E., Jones, G.R. & Cain, K.D. (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. *Journal of Fish Diseases* **26,** 377-384.

LaFrentz B.R., LaPatra, S.E., Jones, G.R., Congleton, J.L., Sun, B. & Cain, K.D. (2002) Characterization of serum and mucosal antibody responses and relative per cent survival in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunization and challenge with *Flavobacterium psychrophilum*. *Journal of Fish Diseases* **25,** 703-713.

Li J., Barreda, D.R., Zhang, Y., Boshra, H., Gelman, A.E., Lapatra, S., Tort, L. & Sunyer, J.O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature immunology* **7,** 1116-1124.

Lieschke G.J. & Trede, N.S. (2009) Fish immunology. *Current Biology* **19,** R678-R682.

Liu H., Izumi, S. & Wakabayashi, H. (2001) Detection of *Flavobacterium psychrophilum* in various organs of ayu *Plecoglossus altivelis* by in situ hybridization. *Fish Pathology* **36,** 7-11.

Lorenzen E. (1994) Studies on *Flexibacter psychrophilus* in relation to rainbow trout fry syndrome (RTFS). Ph.D. thesis, National Veterinary Laboratory, Denmark.

Lorenzen E., Brudeseth, B.E., Wiklund, T. & Lorenzen, N. (2010) Immersion exposure of rainbow trout (*Oncorhynchus mykiss*) fry to wildtype *Flavobacterium psychrophilum* induces no mortality, but protects against later intraperitoneal challenge. *Fish & shellfish immunology* **28,** 440-444.

Lorenzen E., Dalsgaard, I., From, J., Hansen, E.M., Hørlyck, V., Korsholm, H., Mellergaard, S. & Olsen, N.J. (1991) Preliminary investigations of fry mortality syndrome in rainbow trout. *Bulletin of the European Association of Fish Pathologists* **11,** 77-79.

Lorenzen E. & Olesen, N.J. (1997) Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome: II. Serological studies. *Diseases of aquatic organisms* **31,** 209-220.

Lovy J., Wright, G.M. & Speare, D.J. (2008) Comparative Cellular Morphology Suggesting the Existence of Resident Dendritic Cells Within Immune Organs of Salmonids. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* **291,** 456-462.

Luis Martinez J., Casado, A. & Enriquez, R. (2004) Experimental infection of *Flavobacterium psychrophilum* in fins of Atlantic salmon *Salmo salar r*evealed by scanning electron microscopy. *Diseases of aquatic organisms* **59,** 79-84.

Madetoja J., Dalsgaard, I. & Wiklund, T. (2002) Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. *Diseases of aquatic organisms* **52,** 109-118.

Madetoja J., Nyman, P. & Wiklund, T. (2000) Flavobacterium psychrophilum, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. *Diseases of aquatic organisms* **43,** 27-38.

Madetoja J., Nystedt, S. & Wiklund, T. (2003) Survival and virulence of *Flavobacterium psychrophilum* in water microcosms. *FEMS microbiology ecology* **43,** 217-223.

Madsen L. & Dalsgaard, I. (2000) Comparative studies of Danish *Flavobacterium psychrophilum* isolates: ribotypes, plasmid profiles, serotypes and virulence. *Journal of Fish Diseases* **23,** 211-218.

Madsen L. & Dalsgaard, I. (1998) Characterization of *Flavobacterium psychrophilum*; comparison of proteolytic activity and virulence of strains isolated from rainbow trout (*Oncorhynchus mykiss*). *Methodology in Fish Diseases Research (ed. by A.C. Barnes, G.A. Davidson, M.P. Hiney & D. McIntosh)*, pp. 45-52, Fisheries Research Services, Aberdeen, Scotland

Madsen L., Moller, J.D. & Dalsgaard, I. (2005) *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), hatcheries: studies on broodstock, eggs, fry and environment. *Journal of Fish Diseases* **28,** 39-47.

Madsen L. & Dalsgaard, I. (1999) Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. *Diseases of aquatic organisms* **36,** 169-176.

Magnadottir B. (2010) Immunological control of fish diseases. *Marine biotechnology (New York, N.Y.)* **12,** 361-379.

Magnadottir B. (2006) Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20,** 137-151.

Martinéz-Porchas M., Martinéz-Córdova, L.R. & Ramos-Enriquez, R. (2009) Cortisol and Glucose: Reliable indicators of fish stress? *Pan-American Journal of Aquatic Sciences* **4,** 158-178.

Mata M., Skarmeta, A. & Santos, Y. (2002) A proposed serotyping system for *Flavobacterium psychrophilum*. *Letters in applied microbiology* **35,** 166.

Ministeriet for Fødevarer, Landbrug og Fiskeri [The Ministry of Food, Agriculture and Fisheries of Denmark] (2013) Fishery Statistics. Retrieved October 8, 2013, from www.naturerhverv.fvm.dk.

Møller J.D. (2004) *Flavobacterium psychrophilum* - surface structures and mechanisms involved n host-pathogen interactions. Ph.D. thesis, Danish Institute for Fisheries Research & The Royal Veterinary and Agricultural University, Denmark.

Murphy K., Travers P. & Walport M. (2008) *Janeway's Immunobiology*, 7th edn. Garland Science, Taylor and Francis Group, LLC, New York and London.

Narnaware Y.K., Baker, B.I. & Tomlinson, M.G. (1994) The effect of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry* **13,** 31-40.

Nematollahi A., Decostere, A., Pasmans, F., Ducatelle, R. & Haesebrouck, F. (2003) Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss* . *Diseases of aquatic organisms* **55,** 101-107.

Nematollahi A., Decostere, A., Pasmans, F. & Haesebrouck, F. (2003) *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases* **26,** 563-574.

Nematollahi A., Pasmans, F., Haesebrouck, F. & Decostere, A. (2005) Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. *Diseases of aquatic organisms* **64,** 23-28.

Nilsen H., Olsen, A.B., Vaagnes, Ø., Hellberg, H., Bottolfsen, K., Skjelstad, H. & Colquhoun, D.J. (2011) Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. *Journal of Fish Diseases* **34,** 403-408.

Obach A. & Laurencin, F.B. (1991) Vaccination of Rainbow Trout *Oncorhynchus Mykiss* Against the Visceral Form Of Coldwater Disease. *Diseases of aquatic organisms* **12,** 13-16.

Ohtani M., Hayashi, N., Hashimoto, K., Nakanishi, T. & Dijkstra, J.M. (2008) Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. *Immunogenetics* **60,** 383-39

Ohtani M. & Raida, M.K. (2013) *Yersinia ruckeri* O1 infect rainbow trout (*Oncorhynchus mykiss*) through the gills demonstrated by three-dimensional imaging analysis called optical projection tomography. *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 88.

Olsen M.M., Kania, P.W., Heinecke, R.D., Skjoedt, K., Rasmussen, K.J. & Buchmann, K. (2011) Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: Molecular and immunohistochemical studies. *Fish & shellfish immunology* **30,** 859-869.

Orieux N., Bourdineaud, J., Douet, D., Daniel, P. & Le Hénaff, M. (2011) Quantification of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), tissues by qPCR. *Journal of Fish Diseases* **34,** 811-821.

Orieux N., Douet, D.G., Le Henaff, M. & Bourdineaud, J.P. (2013) Prevalence of *Flavobacterium psychrophilum* bacterial cells in farmed rainbow trout: Characterization of metallothionein A and interleukin1-beta genes as markers overexpressed in spleen and kidney of diseased fish. *Veterinary microbiology* **162,** 127-135.

Ostland V.E., McGrogan, D.G. & Ferguson, H.W. (1997) Cephalic osteochondritis and necrotic scleritis in intensively reared salmonids associated with *Flexibacter psychrophilus*. *Journal of Fish Diseases* **20,** 443-451.

Overturf K. & LaPatra, S. (2006) Quantitative expression (Walbaum) of immunological factors in rainbow trout, *Oncorhynchus mykiss* (Walbaum), after infection with either *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, or infectious haematopoietic necrosis virus. *Journal of Fish Diseases* **29,** 215-224.

Pacha R.E. (1968) Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. *Applied Microbiology* **16,** 97-101.

Palti Y. (2011) Toll-like receptors in bony fish: from genomics to function. *Developmental and comparative immunology* **35,** 1263-1272.

Parham P. & Janeway C. (2009) *The immune system*. Garland Science, London, New York.

Pedersen, L-F. (2010) *Undersøgelse af miljøvenlige dambrugshjælpestoffer til erstatning for formalin*, DTU Aqua-rapport no. 218-2010, DTU Aqua, Charlottenlund, Denmark.

Pickering A.D. (1992) Rainbow trout husbandry: management of the stress response. *Aquaculture* **100,** 125-139.

Plant K.P., LaPatra, S.E. & Cain, K.D. (2009) Vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with recombinant and DNA vaccines produced to *Flavobacterium psychrophilum* heat shock proteins 60 and 70. *Journal of Fish Diseases* **32,** 521-534.

Rach J.J., Schreier, T.M., Howe, G.E. & Redman, S.D. (1997) Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *Progressive Fish-Culturist* **59,** 41-46.

Raida M.K. & Buchmann, K. (2008a) Bath vaccination of rainbow trout (*Oncorhynchus mykiss* Walbaum) against *Yersinia ruckeri*: Effects of temperature on protection and gene expression. *Vaccine* **26,** 1050-1062.

Raida M.K. & Buchmann, K. (2008b) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri* . *Fish & shellfish immunology* **25,** 533-541.

Raida M.K. & Buchmann, K. (2007) Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination. *Diseases of aquatic organisms* **77,** 41-52.

Raida M.K. & Buchmann, K. (2009) Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. *Developmental and comparative immunology* **33,** 35-45.

Ramirez-Carrozzi V., Sambandam, A., Luis, E., Lin, Z., Jeet, S., Lesch, J., Hackney, J., Kim, J., Zhou, M., Lai, J., Modrusan, Z., Sai, T., Lee, W., Xu, M., Caplazi, P., Diehl, L., de Voss, J., Balazs, M., Gonzalez, L.,Jr, Singh, H., Ouyang, W. & Pappu, R. (2011) IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. *Nature immunology* **12,** 1159-1166.

Ramirez-Gomez F., Greene, W., Rego, K., Hansen, J.D., Costa, G., Kataria, P. & Bromage, E.S. (2012) Discovery and characterization of secretory IgD in rainbow trout: secretory IgD is produced through a novel splicing mechanism. *Journal of immunology (Baltimore, Md.: 1950)* **188,** 1341-1349.

Randelli E., Buonocore, F. & Scapigliati, G. (2008) Cell markers and determinants in fish immunology. *Fish & shellfish immunology* **25,** 326-340.

Rangdale R.E. (1995) Studies on Rainbow Trout fry Syndrome (RTFS). Ph.D. thesis, University of Stirling, Scotland

Rangdale R.E., Richards, R.H. & Alderman, D.J. (1999) Histopathological and electron microscopical observations on rainbow trout fry syndrome. *The Veterinary record* **144,** 251-254.

Rauta P.R., Nayak, B. & Das, S. (2012) Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. *Immunology letters* **148,** 23-33.

Roberts R. (1989) *Fish Pathology*. 2nd Ed., Bailiere Tindall, London, U.K.

Roque A., Yildiz, H.Y., Carazo, I. & Duncan, N. (2010) Physiological stress responses of sea bass (*Dicentrarchus labrax*) to hydrogen peroxide (H_2O_2) exposure. *Aquaculture* **304,** 104-107.

Schmidt A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K. & Larsen, J.L. (2000) Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Applied and Environmental Microbiology* **66,** 4908-4915.

Schreck C.B. (2010) Stress and fish reproduction: the roles of allostasis and hormesis. *General and comparative endocrinology* **165,** 549-556.

Secades P., Alvarez, B. & Guijarro, J.A. (2003) Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium psychrophilum*. *FEMS microbiology letters* **226,** 273-279.

Seppola M., Larsen, A.N., Steiro, K., Robertsen, B. & Jensen, I. (2008) Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). *Molecular immunology* **45,** 887-897.

Sortkjær, O., Bovbjerg, P., Steenfeldt, S.J., Bruun, M.S., Dalsgaard, I., Nielsen, P. & Aarup, P. (2000) *Undersøgelse af eventuelle miljøpåvirkninger ved anvendelse af hjælpestoffer og medicin i ferskvandsdambrug samt metoder til at reducere/eliminere sådanne påvirkninger*, DFU-rapport, no. 79-00, Danmarks Fiskeriundersøgelser, Charlottenlund. Denmark.

Thomas-Jinu S. & Goodwin, A.E. (2004) Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. *Journal of Fish Diseases* **27,** 23-28.

Tidwell J.H. & Allan, G.L. (2001) Fish as food: aquaculture's contribution. *EMBO reports* **2,** 958-963.

Tobback E., Decostere, A., Hermans, K., Ryckaert, J., Duchateau, L., Haesebrouck, F. & Chiers, K. (2009) Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout *Oncorhynchus mykiss. Diseases of aquatic organisms* **84,** 219-228.

Tort M.J., Jennings-Bashore, C., Wilson, D., Wooster, G.A. & Bowser, P.R. (2002) Assessing the effects of increasing hydrogen peroxide dosage on rainbow trout gills utilizing a digitized scoring methodology. *Journal of Aquatic Animal Health* **14,** 95-103.

Toyama T., Kita-Tsukamoto, K. & Wakabayashi, H. (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. *Fish Pathology* **29,** 271-275.

Tubbs L., Wybourne, B.A. & Lumsden, J.S. (2010) Nodular gill disease causing proliferative branchitis and mortality in Chinook salmon (*Oncorhynchus tshawytscha*). *New Zealand veterinary journal* **58,** 59-61.

Uchida D., Hirose, H., Chang, P.K., Aranishi, F., Hirayabu, E., Mano, N., Mitsuya, T., Prayitno, S.B. & Natori, M. (2000) Characterization of Japanese eel immunoglobulin M and its level in serum. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **127,** 525-532.

Vatsos I.N., Thompson, K.D. & Adams, A. (2003) Starvation of *Flavobacterium psychrophilum* in broth, stream water and distilled water. *Diseases of aquatic organisms* **56,** 115-126.

Villarroel F., Zambrano, A., Amthauer, R. & Concha, M.I. (2009) Detection of up-regulated serum amyloid A transcript and of amyloid AA aggregates in skeletal muscle lesions of rainbow trout infected with *Flavobacterium psychrophilum* . *Veterinary immunology and immunopathology* **130,** 120-124.

Villarroel F., Casado, A., Vásquez, J., Matamala, E., Araneda, B., Amthauer, R., Enriquez, R. & Concha, M.I. (2008) Serum amyloid A: A typical acute-phase reactant in rainbow trout? *Developmental and comparative immunology* **32,** 1160-1169.

Wang T., Bird, S., Koussounadis, A., Holland, J.W., Carrington, A., Zou, J. & Secombes, C.J. (2009) Identification of a novel IL-1 cytokine family member in teleost fish. *Journal of Immunology* **183,** 962-974.

Wang T., Martin, S.A.M. & Secombes, C.J. (2010) Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated. *Developmental & Comparative Immunology* **34,** 491-500.

Wang T., Monte, M.M., Huang, W., Boudinot, P., Martin, S.A.M. & Secombes, C.J. (2010) Identification of two FoxP3 genes in rainbow trout (*Oncorhynchus mykiss*) with differential induction patterns. *Molecular immunology* **47,** 2563-2574.

Watts M., Munday, B. & Burke, C. (2001) Immune responses of teleost fish. *Australian Veterinary Journal* **79,** 570-574.

Wiklund T., Madsen, L., Bruun, M.S. & Dalsgaard, I. (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. *Journal of applied microbiology* **88,** 299-307.

Wiklund T. & Dalsgaard, I. (2002) Survival of *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) serum in vitro. *Fish & shellfish immunology* **12,** 141-153.

Wilson J.M. & Laurent, P. (2002) Fish gill morphology: inside out. *Journal of Experimental Zoology* **293,** 192-213.

Wilson M., Henderson, B. & McNab, R. (2002) Bacterial disease mechanisms: An introduction to cellular microbiology. Wiley, Chichester, U.K.

Zapata A.G. & Cooper E.L. (1990) *The Immune System: Comparative Histophysiology*. Wiley, Chichester, U.K.

Zhang Y., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., Lapatra, S.E., Bartholomew, J. & Sunyer, J.O. (2010) IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature immunology* **9**, 827-835.

Zhang Z., Chi, H., Niu, C., Bogwald, J. & Dalmo, R.A. (2011) Molecular cloning and characterization of Foxp3 in Atlantic salmon (*Salmo salar*). *Fish & shellfish immunology* **30,** 902-909.

Zhu L.Y., Pan, P.P., Fang, W., Shao, J.Z. & Xiang, L.X. (2012) Essential role of IL-4 and IL-4Rα interaction in adaptive immunity of zebrafish: insight into the origin of Th2-like regulatory mechanism in ancient vertebrates. *Journal of immunology (Baltimore, Md.: 1950)* **188,** 5571-5584.

Zwollo P., Cole, S., Bromage, E. & Kaattari, S. (2005) B cell heterogeneity in the teleost kidney: Evidence for a maturation gradient from anterior to posterior kidney. *Journal of Immunology* **174,** 6608-6616.

Presentations

2011

Challenge models for RTFS in rainbow trout fry (*Oncorhynchus mykiss*).
Oral presentation at DAFINET Workshop, May 3-5 2011, Svaneke, Denmark. Contributors: Henriksen*, Maya Maria Mihályi; Madsen, Lone; Dalsgaard, Inger.

***Flavobacterium psychrophilum*, prevention and immune response.**
Oral and poster presentation at 12th Fish Immunology Workshop, April 17-21 2011, Wageningen, Netherlands. Contributors: Henriksen*, Maya Maria Mihályi; Dalsgaard, Inger.

Immune response in rainbow trout against infection with *Flavobacterium psychrophilum*.
Poster presentation (nr. 121, p. 295) at 15th International Conference on Diseases of Fish and Shellfish, September 12-16, Split, Croatia. Contributors: Henriksen*, Maya Maria Mihályi; Kania, Per Walter; Madsen, Lone; Buchmann, Kurt; Dalsgaard, Inger.

2012

Immersion challenge with *Flavobacterium psychrophilum* in rainbow trout fry (*Oncorhynchus mykiss*).
Oral presentation at DAFINET Workshop, April 17-21 2012, Copenhagen, Denmark. Contributors: Henriksen*, Maya Maria Mihályi; Madsen, Lone; Dalsgaard, Inger.

Current research on *Flavobacterium psychrophilum*.
Oral presentation at Flavobacterium 2012, June 5-7, Åbo/Turku, Finland. Contributors: Dalsgaard*, Inger; Henriksen, Maya Maria Mihályi; Christiansen, Rói Hammershaimb; Madsen, Lone.

Hydrogen peroxide as pre-treatment stressor in experimental immersion challenge of rainbow trout fry with *Flavobacterium psychrophilum*.
Oral presentation at Flavobacterium 2012, June 5-7, Åbo/Turku, Finland. Contributors: Henriksen*, Maya Maria Mihályi; Madsen, Lone; Dalsgaard, Inger.

The role of the gills as potential portal of entry in rainbow trout fry syndrome.
Oral presentation at DAFINET Workshop: Immune Responses in Fish, November 6-7, Copenhagen, Denmark. Contributors: Henriksen*, Maya Maria Mihályi; Kania, Per Walther; Madsen, Lone; Buchmann, Kurt; Dalsgaard, Inger.

2013

Immune response in the head kidney of rainbow trout fry following stress and infection with *Flavobacterium psychrophilum*.
Poster presentation (nr. 264, p. 410) at 16th International Conference on Diseases of Fish and Shellfish, September 2-6, Tampere, Finland. Contributors: Henriksen*, Maya Maria Mihályi; Kania, Per Walter; Madsen, Lone; Buchmann, Kurt; Dalsgaard, Inger.

Accompanying papers

PAPER I

OPEN ACCESS Freely available online

PLOS ONE

Effect of Hydrogen Peroxide on Immersion Challenge of Rainbow Trout Fry with *Flavobacterium psychrophilum*

Maya Maria Mihályi Henriksen*, Lone Madsen, Inger Dalsgaard

Technical University of Denmark, National Veterinary Institute, Bülowsvej 27, Frederiksberg C, Denmark

Abstract

An experimental model for immersion challenge of rainbow trout fry (*Oncorhynchus mykiss*) with *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome and bacterial cold water disease was established in the present study. Although injection-based infection models are reliable and produce high levels of mortality attempts to establish a reproducible immersion model have been less successful. Various concentrations of hydrogen peroxide (H_2O_2) were evaluated before being used as a pre-treatment stressor prior to immersion exposure to *F. psychrophilum*. H_2O_2 accelerated the onset of mortality and increased mortality approximately two-fold; from 9.1% to 19.2% and from 14.7% to 30.3% in two separate experiments. Clinical signs observed in the infected fish corresponded to symptoms characteristically seen during natural outbreaks. These findings indicate that pre-treatment with H_2O_2 can increase the level of mortality in rainbow trout fry after exposure to *F. psychrophilum*.

Citation: Henriksen MMM, Madsen L, Dalsgaard I (2013) Effect of Hydrogen Peroxide on Immersion Challenge of Rainbow Trout Fry with *Flavobacterium psychrophilum*. PLoS ONE 8(4): e62590. doi:10.1371/journal.pone.0062590

Editor: Dongsheng Zhou, Beijing Institute of Microbiology and Epidemiology, China

Received December 14, 2012; **Accepted** March 22, 2013; **Published** April 30, 2013

Funding: This work was performed as a part of the Danish Fish Immunology Research Centre and Network (DAFINET; www.dafinet.dk) supported by the Danish Council of Strategic Research (grant 2101-080017). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mmah@vet.dtu.dk

Introduction

Flavobacterium psychrophilum is a Gram-negative fish pathogen and the causative agent of bacterial cold water disease (BCWD), also called rainbow trout fry syndrome (RTFS). Since first being discovered in USA in the 1940s, the disease has spread worldwide and was identified in Western Europe in the mid 1980s. Today it has a significant impact on rainbow trout (*Oncorhynchus mykiss*) and other salmonid fish in aquaculture worldwide [1]. Clinical signs of the disease include a dark coloration of the skin, pale gills and organs (kidney, intestine and liver) due to anemia. A loss of appetite and lethargic behavior is also observed.

Injection-based experimental challenges with *F. psychrophilum* have been standardized and the resulting mortality depends on several factors, including the number of colony forming units (CFU) injected, size of the fish, batch differences and the number of fish in each tank [2]. However, infection by injection is not a suitable approach for all investigations since the first line of defense, consisting of non-specific barriers, are bypassed. It has proven difficult to produce high and consistent mortality rates using cohabitation and immersion models unless stress or scarification has been applied [2–7], while immersion exposure to *F. psychrophilum* in the logarithmic phase of growth reportedly results in significant mortality rates [8].

Bath-treatment with various non-medical compounds, such as copper sulphate, chloramine-T, sodium carbonates, sodium chloride, formalin and hydrogen peroxide (H_2O_2), are routinely used against pathogens in aquaculture [9]. One previous immersion model for *F. psychrophilum* used formalin as stressor, which raised the mortality rate significantly [2]. Since the use of formalin is to be phased out by Danish fish farmers due to human health considerations, another less harmful stressor is needed. H_2O_2 is a useful and environmentally friendly alternative to formalin and has been used against skin parasites, bacterial gill infections and mold on eggs in Denmark for over ten years [10,11]. The use of H_2O_2 has been shown to accelerate *Tenacibaculum maritimum* infections in turbot [12] and *Flavobacterium columnaris* infections in channel catfish [13]. Accordingly, H_2O_2 was an obvious candidate for a stressor in a *F. psychrophilum* immersion model.

The aim of this study was to establish a reproducible method for increasing mortality of RTFS in immersion challenge of rainbow trout fry. A reliable model is needed for studies on pathogen invasion, host immune response and efficacy of treatments. First, various concentrations of H_2O_2 were tested in experiment 1 before being combined with immersion exposure to a virulent *F. psychrophilum* strain at two different phases of bacterial growth in experiment 2. The most favorable H_2O_2 concentration and bacterial growth phase were combined in experiment 3, after which the temperature and number of animals were adjusted in experiment 4 to increase the power of the model.

Materials and Methods

Fish and Rearing Conditions

Three batches of eggs from the same stock and family of fish were used in all four experiments (see Table 1). The eggs originated from Fousing Trout Farm (Denmark) and disinfection and hatching was carried out at AquaBaltic (Bornholm, Denmark). The fish were acclimatized for at least 3 weeks in 200 L tanks containing 15°C recirculated tap water (non-chlorinated), while nitrate, nitrite and ammonia levels were monitored continuously.

The fish were fed dry commercial feed daily (INICIO Plus, BioMar A/S, Denmark). All tanks, except the 1 and 2 L tanks used during challenge and pre-treatment, were opaque in order to minimize external stress due to movement in the experimental facility. Upon arrival, at least 10 fish from each batch where sacrificed by an overdose of MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma), examined for *F. psychrophilum* and proven to be free of the pathogen. Samples from brain and kidney were streaked on tryptone yeast extract salts (TYES) and blood agar plates, while spleen samples were placed in TYES broth (110 rpm). Samples were incubated for 7 d at 15°C for TYES broth/plates and at 20°C for blood plates. Yellow colonies were further examined by species specific PCR. First, a pure culture was obtained by streaking and after 5–7 d of growth, a single colony was suspended in 50 µL distilled water and boiled for 5 min to lyse the bacterial cells and placed on ice. Then, species specific primers, referred to as PSY1 and PSY2, amplifying the 16S rRNA genes of *F. psychrophilum* were used [14] for PCR amplifications, which were carried out according to a previously described method [15]. In brief, Ready to Go PCR beads (cat. no. 27955901, Amersham Pharmacia Biotech, Millwaukee, USA), including all reagents, were used. Ten pmol of each primer and 1 µL of the lysed bacteria were added to each reaction. Distilled water was added to a total volume of 25 µL. Amplifications were carried out using a Biometra T- 3000 thermocycler amplifier (Biometra, Göttingen, Germany): 95°C for 5 min followed by 35 cycles consisting of 30 s at 95°C, 30 s at 57°C and 60 s at 72°C.

Before randomly selecting the experimental groups, the largest (approx. >1.5 g) and smallest (approx. <0.6 g) fish were removed in order to minimize size variation. The fish were inspected at least once every 24 h and dead fish were examined for *F. psychrophilum* as previously described. Fish surviving until the completion of each experiment were euthanized using an overdose of MS-222.

The described work was carried out in accordance with the internationally accepted guidelines for care and use of laboratory animals in research and procedures were approved by the Committee for Animal Experimentation, Ministry of Justice, Copenhagen, Denmark (J.nr. 2006/561-1204 and 2011/561-51). During experiments, the fish were inspected several times a day and precautions were taken to minimize stress by keeping the fish in opaque tanks, minimizing handling and monitoring water chemistry.

Bacterial Strain and Challenge Dose

A Danish *F. psychrophilum* strain, 950106-1/1, was used for all challenges (serotype Fd and elastin degrading). It was isolated from a clinical outbreak of RTFS in a freshwater farm in 1995, and has previously been used for several i.p. and immersion challenges [2]. The bacterial strain was stored at -80°C in TYES broth with 15–20% glycerol and pre-cultivated in 10 mL TYES broth at 15°C for 3 d (110 rpm). Then, 100 mL TYES broth was inoculated using 0.5 mL of the pre-culture and optical density (OD) was measured at 525 nm using a spectrophotometer (Shimadzu UV-1201) at regular intervals to establish a growth curve. Logarithmic and stationary phases were determined after 24 or 48 h of growth, respectively. Before being used for challenge, each culture was examined microscopically to assert purity and CFU counts were carried out by spreading 0.1 mL of 10-fold dilutions (10^{-4} to 10^{-7}) on TYES agar in duplicates.

Experiment 1: H_2O_2 as Pre-treatment Stressor

Three groups of 6 fish in duplicates (n = 36) were immersed for 60 min in aerated 1 L tanks containing final concentrations of 50, 150 or 300 mg H_2O_2 L^{-1} (prod. no. 1072090250, Merck KGaA, Germany). H_2O_2 was added directly into the tanks already containing the fish. Subsequently, the fish were removed and placed in 20 L aquaria and observed for 14 d. The temperature during the experiment was 15±0.5°C, while H_2O_2 treatment was carried out at approximately 12°C. Based on the results, a concentration of 200 mg H_2O_2 L^{-1} was chosen as stressor for experiment 2.

Experiment 2: H_2O_2 Combined with Bacterial Growth Phases (24 and 48 h)

Four groups of 10 fish in duplicates (n = 80) were immersed for 60 min in aerated 1 L tanks containing 200 mg H_2O_2 L^{-1} or kept under similar conditions in water. H_2O_2 was added directly into the aquaria already containing the fish. Afterwards, the fish were removed and placed for 30 min in a tank containing a 1:9 dilution of cultures grown for either 24 h or 48 h. This resulted in bacterial concentrations of 10^5 and 10^7 CFU mL^{-1} water, respectively. Finally, the fish were placed in 20 L aquaria containing tap water. The fish were observed for 30 d. The temperature during the experiment was 14.5±0.5°C, while H_2O_2 treatment and bacterial challenge were carried out at a temperature below 14°C. The animals dying after exposure to the pathogen were sampled as previously described for the bacteriological examination. Since no significant difference regarding either mortality or reisolation of the pathogen was observed, the 48 h culture was chosen for the following experiments, since it has been used successfully in previous investigations [2].

Experiment 3:150 mg L^{-1} H_2O_2 as Experimental Stressor

Four groups of 50 fish in duplicates (n = 400) were immersed for 60 min in aerated 2 L tanks containing 150 mg H_2O_2 L^{-1} or kept under similar conditions in tap water. H_2O_2 was diluted in 300 mL tap water, before being added to the aquaria already containing the fish. Subsequently, the fish were removed and immersed in a 1:9 diluted broth culture (48 h) containing 10^7 CFU mL^{-1} for 30 min, while controls were immersed in

Table 1. Experimental parameters.

Exp.	Batch	Weight	Length	H_2O_2 mg L^{-1}	*F.p* CFU mL^{-1}	°C	n (pr. replicate)
1	1	0.69 g (±0.21)	4.4 cm (±0.3)	50/150/300	–	15.5±1.0	36 (6)
2	1	0.77 g (±0.18)	4.4 cm (±0.3)	−/200	10^5(24 h)/10^7(48 h)	16.0±0.5	80 (10)
3	2	1.2 g (±0.50)	5.0 cm (±0.8)	−/150	−/10^7	16.8±0.5	400 (50)
4	3	1.1 g (±0.34)	4.7 cm (±0.5)	−/200	−/10^7	14.0±0.5	500 (50)

Fish batches and mean values for weight, length and temperature are stated along with standard deviation (SD). The applied H_2O_2 concentrations are given in mg L^{-1}, while the final bacterial concentrations in broth used for immersion challenge are stated in CFU mL^{-1}. The symbol '−' designates the absence of *F.p.* and H_2O_2.
doi:10.1371/journal.pone.0062590.t001

sterile broth. Finally, the fish were placed in 30 L aquaria and mortality observed for 50 d. The temperature during the experiment was 16.8±0.5°C, while H_2O_2 treatment and bacterial challenge were carried out at a temperature below 14°C. To increase mortality in experiment 4, the temperature was lowered and the concentration of H_2O_2 increased.

Experiment 4:200 mg L^{-1} H_2O_2 as Experimental Stressor

Four groups of 50 fish in duplicates for uninfected or triplicates for infected (n = 500) were immersed for 60 min in aerated 2 L tanks containing 200 mg H_2O_2 L^{-1} or kept under similar conditions in tap water. H_2O_2 was diluted in 300 mL tap water, before being added to the treatment tanks already containing the fish. Subsequently, the fish were removed and immersed in a 1:9 diluted broth culture (48 h) containing 10^7 CFU mL^{-1} for 30 min, while controls were immersed in sterile broth. Finally, the fish were placed in 30 L aquaria and observed for 40 d. The temperature during the experiment was 14±0.5°C, while H_2O_2 treatment and bacterial challenge were carried out at a temperature below 14°C. Conditions during treatments of the various groups were kept as identical as possible with the same number of nettings and amount of retention time in the 2 L tanks during treatments.

Statistical Analysis

Mortality data was analyzed using the generalized linear model (GLM) on the R software platform [16] and figures have been produced using GraphPad Prism 5. The relative standard deviation (RSD = (standard deviation/mean)*100) was also calculated for mortality rates of infected groups in experiment 3 and 4. Weight and length of the fish was compared between groups (where a sufficient n was available) by using 1-way ANOVA combined with Tukey's test.

In tables and figures, significance levels are denoted by * for p = 0.05, ** for p = 0.01 and *** for p = 0.001.

Results

Hydrogen peroxide pre-treatment was found to elevate the mortality of subsequent immersion exposure to *F. psychrophilum* in the stationary phase of growth.

Experiment 1: H_2O_2 as Pre-treatment Stressor

An apparent escape response was observed in all groups immediately after exposure to H_2O_2; the majority of fish clumped together and swam towards the tank wall. This was most prominent in tanks treated with medium and high concentrations, where the escape response was followed by fish laying on bottom of the tank. Furthermore, a few fish from the high dose group showed erratic swimming behavior for shorter periods of time. All visual changes in behavior ceased after a few min at the lowest concentration, while persisting longer in groups treated with higher concentrations of H_2O_2. All fish survived the treatment, although one death occurred in the high dosage group within the first 24 h.

Experiment 2:200 mg L^{-1} H_2O_2 Combined with Bacterial Growth Phases (24 and 48 h)

The experiment included four groups in duplicates: (1) 24 h culture, (2) 24 h culture with H_2O_2 pre-treatment, (3) 48 h culture and (4) 48 h culture with H_2O_2 pre-treatment (Table 2).

Nine of the 40 fish pre-treated with H_2O_2 died during treatment and were accounted for as acute mortality. It was only possible to re-isolate *F. psychrophilum* from dead fish belonging to groups pre-treated with H_2O_2.

Table 2. H_2O_2 combined with bacterial growth phases.

Group	H_2O_2	*F.p* (CFU mL^{-1})	n	Re-isolation/ dead fish	% mortality
1	–	**24 h**	20	0/3	15%
2	+	(10^5)	12	3/3	25%
3	–	**48 h**	20	0/1	5%
4	+	(10^7)	19	3/4	21%

Experiment 2. The symbols '+' and '-' designate addition or absence of the two factors, H_2O_2 (200 mg L^{-1} for 60 min). and *F.p.* (10^5/10^7 CFU mL^{-1} *F. psychrophilum* for 30 min). Successful reisolations of *F. psychrophilum* from dead fish are given as a fraction of the total number of dead fish. Finally, cumulative mortality is stated in percent.
doi:10.1371/journal.pone.0062590.t002

Experiment 3:150 mg L^{-1} H_2O_2 as Experimental Stressor

The experiment consisted of four groups in duplicates: (I) untreated control, (II) H_2O_2, (III) *F. psychrophilum* and (IV) H_2O_2 and *F. psychrophilum* (Table 3).

Seven of the 200 fish pre-treated with 150 mg H_2O_2 L^{-1} died during the treatment or subsequent transfer to tap water and were accounted for as acute mortality. Seven days after challenge, technical problems lead to an increased temperature in the room and flooding of tank III.B. This led to the loss of 23 fish, which were excluded from the experiment, since the event took place before onset of mortality.

The cumulative mortality (Table 3) was higher in both infected groups compared to their respective controls (p = 0.05 for III and p = 0.001 for IV). The cumulative mortality for infected groups was 9.1% for *F. psychrophilum* challenge alone and 19.2% in combination with H_2O_2 (p = 0.001). Relative standard deviation for the two groups was 23% and 39%, respectively. Furthermore, H_2O_2 accelerated the onset of mortality post challenge from 9 to 3 d (Fig. 1).

Experiment 4:200 mg L^{-1} H_2O_2 as Experimental Stressor

The experiment consisted of four groups, which were set up in either duplicates for uninfected or triplicates for infected groups: (A) untreated control and (B) H_2O_2, (C) *F. psychrophilum* and (D) H_2O_2 and *F. psychrophilum* (Table 4).

Five of the 250 fish pre-treated with 200 mg H_2O_2 L^{-1} died during the treatment and subsequent transfer to tap water and were accounted for as acute mortality.

The cumulative mortality was higher in both infected groups compared to their respective controls (p = 0.001). The cumulative mortality (Table 4) of infected groups was 14.7% (n = 22) for *F. psychrophilum* challenge alone and rose to 30.3% (n = 54) in combination with H_2O_2 (p = 0.001). RSD for the two groups was 27% and 38%, respectively. Furthermore, H_2O_2 accelerated the onset of mortality post challenge from 10 to 4 d (Fig. 2). A statistically significant difference in mortality was found between replicate tanks D1 (20.0%) and D2 (42.9%) from group D (p = 0.05), which was taken into consideration in data processing.

Discussion

Several studies have investigated the potential adverse effects of H_2O_2 on salmonids to establish recommendations for safe treatment dosages [10,11,17–20]. Possible damage caused by

Table 3. Challenge after 150 mg L^{-1} H_2O_2.

Group	Replicate	H_2O_2	*F.p.*	n	% mortality (n)	Cumulative % mortality	SD
I	**A**	–	–	45	2.0% (1)	**2.0%**	
	B	–	–	47	2.0% (1)	(a*)	
II	**A**	+	–	49	4.1% (2)	**4.0%**	
	B	+	–	44	4.4% (2)	(b***)	
III	**A**	–	+	49	8.0% (4)	**9.1%**	2.19
	B	–	+	27	11.1% (3)	(a*)(c**)	(23%)
IV	**A**	+	+	49	14.0% (7)	**19.2%**	7.42
	B	+	+	48	24.5% (12)	(b***)(c**)	(39%)

Experiment 3. The fish weighed 1.2 g and the temperature was 16.8±0.5°C. The symbols '+' and '-' designate addition or absence of the two factors, H_2O_2 (150 mg L^{-1} for 60 min) and *F.p.* (10^7 CFU mL^{-1} *F. psychrophilum* for 30 min). The % cumulative mortality is stated for both replicates and groups along with SD and RSD in brackets. Statistical differences are denoted by a letter and * for p = 0.05, ** for p = 0.01 and *** for p = 0.001.
doi:10.1371/journal.pone.0062590.t003

H_2O_2 depends on several factors, including the applied concentration, exposure time, frequency of treatment, life stage of the fish and temperature during treatment. Damage generally consists of injury to the gills and larger fish are more susceptible [19]. Pathological changes may include an increase in epithelial cell granularity, edemas, lamellar fusion, epithelial hyperplasia as well as swelling and lifting of the gill epithelium. The gills are a frequent target in stress responses and of the mentioned lesions can, with the existing knowledge, be induced by many types of environmental stressor; not just H_2O_2 [23]. Treatment is generally discouraged at temperatures above 14–15°C [11,21,22], but temperatures approaching 14°C during H_2O_2 treatment did not result in increased mortality under the conditions applied in the present experiments.

Mortality due to H_2O_2 has been shown to occur within the first days after treatment; predominantly during or within hours of exposure [17,18]. It is difficult to directly compare the published studies regarding H_2O_2, and the performance of pilot studies on small groups of fish are recommended before treating an entire population [18,19].

H_2O_2 as Pre-treatment Stressor

Previous studies have demonstrated that H_2O_2 has a fast short-term stress effect in both Atlantic salmon and sea bass [24,25]. In the present study, an escape response was observed in all fish treated with H_2O_2, although most prominent in the high dose group, in which the only mortality occurred within 24 h after exposure. Based on these results, a concentration of 200 mg H_2O_2 L^{-1} was chosen as stressor for experiment 2, where 9 of the 40 treated fish died from the pre-treatment alone. The increased mortality is likely due to the direct administration of undiluted 35% H_2O_2 into the water. Since the density of fish was higher in experiment 2, the probability of contact with high local concentrations of H_2O_2 before dispersal in the water was

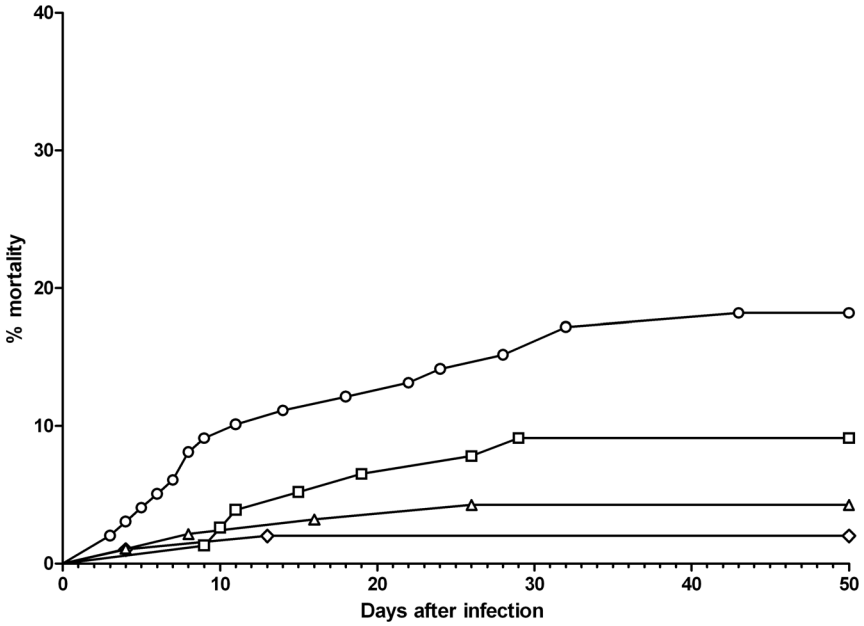


Figure 1. Challenge after 150 mg L^{-1} H_2O_2. Cumulative mortality of the merged replicates is shown in the figure. The fish weighed 1.2 g and the temperature was 16.8±0.5°C. The experiment ran for 50 days. ◇: control, △: H_2O_2, □: *F. psychrophilum*, ○: H_2O_2+ *F. psychrophilum*.
doi:10.1371/journal.pone.0062590.g001

Table 4. Challenge after 200 mg L^{-1} H_2O_2.

Group	Replicate	H_2O_2	*F.p.*	n	% mortality (n)	Cumulative % mortality	SD
A	**1**	–	–	50	0.0% (0)	**3%**	
	2	–	–	50	6.0% (3)	(a***)	
B	**1**	+	–	50	8.0% (4)	**5%**	
	2	+	–	50	2.0% (1)	(b***)	
C	**1**	–	+	50	12.0% (5)	**14.7%**	4.19
	2	–	+	50	14.3% (7)	(a***)(c***)	(27%)
	3	–	+	50	20.0% (10)		
D	**1**	+	+	50	20.0% (10)	**30.3%**	11.57
	2	+	+	49	42.9% (21)	(b***)(c***)	(39%)
	3	+	+	46	28.3% (13)		

Experiment 4. The fish weighed 1.1 g and the temperature was 14±0.5°C. The symbols '+' and '–' designate addition or absence of the two factors, *F.p.* (10^7 CFU mL^{-1} *F. psychrophilum* for 30 min) and H_2O_2 (200 mg L^{-1} for 60 min). The % cumulative mortality is stated for both replicates and groups along with SD and RSD. Statistical differences are denoted by a letter and * for p = 0.05, ** for p = 0.01 and *** for p = 0.001.
doi:10.1371/journal.pone.0062590.t004

increased. In experiment 3 and 4, H_2O_2 was diluted at in least 300 mL water before being added to the treatment tanks. This approach resulted in 2–3.5% acute mortality in addition to 4–5% mortality during the experiments, compared to a total of 2–3% in the untreated control groups. In a previous test, healthy juvenile rainbow trout were treated twice a week for seven weeks (200 mg L^{-1} for 60 min) reported lower feed conversion ratios in the first weeks of treatment but no noteworthy mortality [26], while the same dosage given only once resulted in high mortalities within a few hours under slightly different conditions [17]. The experimental design was not intended to investigate the potential long-term consequences of H_2O_2 treatment, but the results indicate the possibility of excess mortality due to as little as a single treatment but the subject should be investigated further.

H_2O_2 Combined with Bacterial Growth Phases (24 and 48 h)

Experiment 2 was a pilot test used to indicate, whether there was a difference in mortality for fish infected with bacteria in either the logarithmic or stationary phase of growth. Furthermore, it was relevant to conduct a small scale test of H_2O_2 in combination with *F. psychrophilum*. Based on previous results, an increased mortality was expected for the 24 h culture [8], but no differences were seen. Reisolation of *F. psychrophilum* from spleen, kidney and brain of dead fish was only successful in H_2O_2 treated groups. Although the portals of entry have not been determined, *F. psychrophilum* has been found in mucus, fins, gills and stomach of infected fish and it has been speculated, that sub-optimal environmental conditions may allow the bacterium to get across skin and gills [27]. Besides

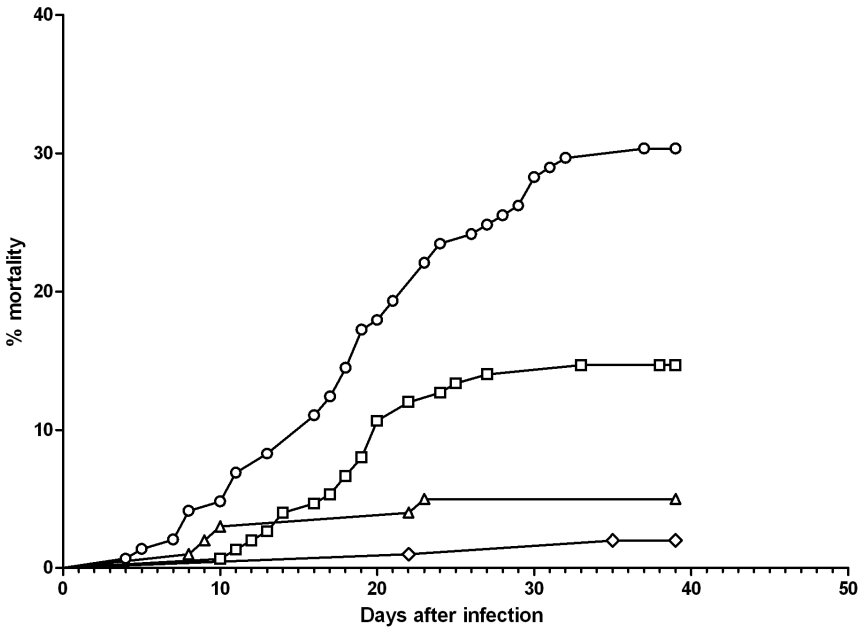


Figure 2. Challenge after 200 mg L^{-1} H_2O_2. Cumulative mortality of the merged replicates is shown in the figure. The fish weighed 1.1 g and the temperature was 14±0.5°C. The experiment ran for 40 days. ◇: control, △: H_2O_2, □: *F. psychrophilum*, ○: H_2O_2+ *F. psychrophilum*.
doi:10.1371/journal.pone.0062590.g002

stressing the host, treatment with H_2O_2 may have resulted in better access to the blood stream, possibly via the gills or by damaging mucus on either skin or gastrointestinal tract, hence allowing for a rapid spread inside the host.

Based on these findings, it was decided to proceed using H_2O_2 as a stressor and the 48 h culture. The 48 h culture has been used successfully in previous investigations [2] and resulted in a higher number of CFU. Cumulative mortality was relatively low for all treatments in experiment 2. The small size of the experimental groups could have affected the outcome in several ways and the results of this experiment cannot be considered to be conclusive. Firstly, the dynamics between healthy, infected and dead fish were influenced by the low density. Secondly, the low number of fish also decreases the statistical robustness, especially since reproducibility is a known problem regarding immersion challenge with *F. psychrophilum*. Choice of strain might have played a role, although 950106-1/1, which was used in the present study, is known to cause mortality in rainbow trout fry [2].

H_2O_2 as Experimental Stressor in Immersion Challenge

The cumulative mortality was significantly increased by pre-treatment with H_2O_2 in both experiment 3 and 4. In experiment 3, pre-treatment with H_2O_2 increased mortality due to *F. psychrophilum* from 9.1% to 19.2%. In experiment 4, the temperature was lowered and the pre-treatment dosage increased slightly, resulting in mortality rates of 14.7% for *F. psychrophilum* alone and 30.3% in combination with H_2O_2. The increased dosage of H_2O_2 may have stressed the fish more or resulted in more damage to gills or other tissues in contact with the surrounding water, such as gastrointestinal tract or skin. The lowered temperature is also likely to have played a role, since even smaller changes have been shown to have significant consequences on survival of infections [27]. At lower temperatures, the immune response of the fish is delayed [28,29], while the psychrophilic bacteria's physiological functions are impaired to a lesser degree [1]. Another explanation is a suppression of the immune system due to cortisol, which is a corticosteroid associated with stress [23]. Finally, batch differences can also have contributed to the difference in mortality seen between the two experiments.

Variation was consistent around 40% for the pre-treated and infected group, and 25% for the infected group for both experiment 3 and 4. Thus, variation did not seem to be influenced by smaller differences in handling through the experiments, since strictly uniformed management was applied in experiment 4. Both variation and proportional change in mortality induced by H_2O_2 was comparable between the two experiments and to the results obtained in a similar model using formalin [2]. Despite the variation, H_2O_2 pre-treatment consistently elevated the mortality after subsequent immersion exposure to *F. psychrophilum*. Conversely, the results underline the importance of maximizing the statistical power of the experimental model by using larger experimental groups or more replicates.

Onset of mortality was accelerated, a pattern which was also observed for H_2O_2 treatment in combination with *T. maritimum* and *F. columnaris* [12,13]. The clinical signs in both experiment 3 and 4 corresponded to the symptoms seen in natural outbreaks and included anemia (resulting in pale gills and organs), splenomegaly (which could in some cases be seen through the skin as a red coloration of the abdomen) and dark pigmentation of the skin [1,30,31].

A number of previous studies have also focused on developing a reliable model for infection, which mimics natural transmission, since it is essential to gain more knowledge regarding transmission and host-pathogen interactions. Previous studies have highlighted the limited pathogenicity of *F. psychrophilum* without using various forms of stress or scarification. A number of studies have not resulted in mortality [4,5,32,33], while infections have been successfully established in other studies [2,7,8,34]. Even when mortality occurred, reproducibility has been a problem [35].

It is hard to directly compare the various studies due to differences in setup; including origin, size and health status of the fish, differences in bacterial strains and growth conditions, method of exposure and diversity in the experimental setup, including the number replicates and animals in each group. Since it is difficult to induce *F. psychrophilum* mortality without using stressors or compromising the outer barriers, it seems unlikely that immersion challenge will be standardized on the same level as seen with injections. Although mortality did not reach 60%, which is the desired goal for testing the potency of fish vaccines [36], the current model seems reproducible, since mortality was elevated in both full scale experiments.

In conclusion, the investigated model seems to be a good alternative to injections for studies requiring natural transmission of the pathogen without bypassing the outer barriers. Although the present study does not deal with the cause for the increased mortality induced by H_2O_2, it emphasizes and allows further investigation of the potential connection between routine non-medical treatments and pathogen outbreaks in aquaculture.

Acknowledgments

The technical assistance provided by Lene Gertman has been greatly appreciated as well as advice given regarding statistics by both Henrik Spliid and Claes Enø. Thanks to Kurt Buchmann and Per Walther Kania from the University of Copenhagen, Department of Veterinary Disease Biology, for allowing the use of their experimental facilities during the first three experiments. Finally, comments and suggestions given by Rune Lindgreen were also greatly appreciated.

Author Contributions

Proofreading: LM ID. Conceived and designed the experiments: MH LM ID. Performed the experiments: MH. Analyzed the data: MH. Contributed reagents/materials/analysis tools: LM ID. Wrote the paper: MH.

References

1. Nematollahi A, Decostere A, Pasmans F, Haesebrouck F (2003) *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases 26: 563–574.
2. Madsen L, Dalsgaard I (1999) Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *oncorhynchus mykiss*. Diseases of Aquatic Organisms 36: 169–176.
3. Borg AF (1960) Studies on myxobacteria associated with diseases in salmonid fishes. American Association for the Advancement of Science 8: 1–85.
4. Madetoja J, Nyman P, Wiklund T (2000) Flavobacterium psychrophilum, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 43: 27–38.
5. Busch S, Dalsgaard I, Buchmann K (2003) Concomitant exposure of rainbow trout fry to *Gyrodactylus derjavini* and *Flavobacterium psychrophilum*: Effects on infection and mortality of host. Veterinary Parasitology 117: 117–122.
6. Holt RA (1988) *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. PhD thesis, Oregon State University, Corvallis.
7. Rangdale RE (1995) Studies on rainbow trout fry syndrome (RTFS). PhD thesis, University of Stirling, Stirling.
8. Aoki M, Kondo M, Kawai K, Oshima S (2005) Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 67: 73–79.

9. Jokumsen A, Svendsen LM (2010) Farming of freshwater rainbow trout in denmark. Charlottenlund. National Institute of Aquatic Resources, Technical University of Denmark. DTU Aqua report no.219–2010.
10. Pedersen LF (2010) Investigations of environmental benign aquaculture therapeutics replacing formalin (in Danish). DTU Aqua, Charlottenlund. DTU Aqua report, no.218–2010.
11. Sortkjær O, Bovbjerg P, Steenfeldt SJ, Bruun MS, Dalsgaard I, et al. (2000) Undersøgelse af eventuelle miljøpåvirkninger ved anvendelse af hjælpestoffer og medicin i ferskvandsdambrug samt metoder til at reducere/eliminere sådanne påvirkninger [in Danish]. DFU report 79–00: 206.
12. Avendaño-Herrera R, Magariños B, Irgang R, Toranzo AE (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). Aquaculture 257: 104–110.
13. Thomas-Jinu S, Goodwin AE (2004) Acute columnaris infection in channel catfish, Ictalurus punctatus (Rafinesque): Efficacy of practical treatments for warmwater aquaculture ponds. Journal of Fish Diseases 27: 23–28.
14. Toyama T, Kita-Tsukamoto K, Wakabayashi H (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. Fish Pathology 29: 271–275.
15. Wiklund T, Madsen L, Bruun MS, Dalsgaard I (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. Journal of Applied Microbiology 88: 299–307.
16. R Development Core Team (2011) R: A language and environment for statistical computing, reference index version 2.13.0. R Foundation for Statistical Computing, Vienna.
17. Tort MJ, Jennings-Bashore C, Wilson D, Wooster GA, Bowser PR (2002) Assessing the effects of increasing hydrogen peroxide dosage on rainbow trout gills utilizing a digitized scoring methodology. Journal of Aquatic Animal Health 14: 95–103.
18. Gaikowski MP, Rach JJ, Ramsay RT (1999) Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. Aquaculture 178: 191–207.
19. Rach JJ, Schreier TM, Howe GE, Redman SD (1997) Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. Progressive Fish-Culturist 59: 41–46.
20. Arndt RE, Wagner EJ (1997) The toxicity of hydrogen peroxide to rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clark*i fry and fingerlings. Journal of the World Aquaculture Society 28: 150–157.
21. Harper C, Wolf JC (2009) Morphologic effects of the stress response in fish. Ilar Journal 50: 387–396.
22. Kiemer MCB, Black KD (1997) The effects of hydrogen peroxide on the gill tissues of atlantic salmon, *Salmo salar* L. Aquaculture 153: 181–189.
23. Bruno DW, Raynard RS (1994) Studies on the use of hydrogen peroxide as a method for the control of sea lice on atlantic salmon. Aquaculture International 2: 10–18.
24. Bowers JM, Speare DJ, Burka JF (2002) The effects of hydrogen peroxide on the stress response of Atlantic salmon (*salmo salar*). J Journal of Veterinary Pharmacology and Therapeutics 25: 311–313.
25. Roque A, Yildiz HY, Carazo I, Duncan N (2010) Physiological stress responses of sea bass (*Dicentrarchus labrax*) to hydrogen peroxide (H_2O_2) exposure. Aquaculture 304: 104–107.
26. Speare DJ, Arsenault GJ (1997) Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 54: 2653–2658.
27. Nematollahi A, Decostere A, Pasmans F, Haesebrouck F (2003) *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Disease 26: 563–574.
28. Covert JB, Reynolds WW (1977) Survival value of fever in fish. Nature 267: 43–45.
29. Raida MK, Buchmann K (2007) Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination. Diseases of Aquatic Organisms 77: 41–52.
30. Gräns A, Rosengren M, Niklasson L, Axelsson M (2012) Behavioural fever boosts the inflammatory response in rainbow trout *Oncorhynchus mykiss*. Journal of Fish Biology 81: 1111–1117.
31. Lorenzen E, Dalsgaard I, From J, Hansen EM, Hørlyck V, et al. (1991) Preliminary investigations of fry mortality syndrome in rainbow trout. Bulletin of the European Association of Fish Pathologists 11: 77–79.
32. Bruno DW (1992) *Cytophaga psychrophila* (='*Flexibacter psychrophilus*') (borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the UK. Eur Assoc Fish Pathol 12: 215–216.
33. Decostere A, Lammens M, Haesebrouck F (2000) Difficulties in experimental infection studies with *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) using immersion, oral and anal challenges. Research in Veterinary Science 69: 165–169.
34. Chua FHC (1991) A study on the rainbow trout fry syndrome. MSc thesis, Institute of Aquaculture, University of Stirling, Stirling.
35. Garcia C, Pozet F, Michel C (2000) Standardization of experimental infection with *Flavobacterium psychrophilum*, the agent of rainbow trout *Oncorhynchus mykiss* fry syndrome. Diseases of Aquatic Organisms 42: 191–197.
36. Amend DF (1981) Potency testing of fish vaccines. Developments in Biological Standardization 49: 447–454.

PAPER II

 doi:10.1111/jfd.12201

Evaluation of the immune response in rainbow trout fry, *Oncorhynchus mykiss* (Walbaum), after waterborne exposure to *Flavobacterium psychrophilum* and/or hydrogen peroxide

M M M Henriksen[1], P W Kania[2], K Buchmann[2] and I Dalsgaard[1]

1 Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark

2 Laboratory of Aquatic Pathobiology, Faculty of Health and Medical Sciences, Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg C, Denmark

Abstract

The immune response in rainbow trout fry against *Flavobacterium psychrophilum* was elucidated using an immersion-based challenge with or without prior exposure to hydrogen peroxide (H_2O_2). Samples were taken from the head kidney 4, 48, 125 and 192 h after immersion, and the regulation of several genes was examined. Bacterial load was assessed based on the presence of 16S rRNA and correlated with gene expression, and the levels of specific antibodies in the blood were measured 50 days post-infection. Separately, both H_2O_2 and *F. psychrophilum* influenced gene expression, and pre-treatment with H_2O_2 influenced the response to infection with *F. psychrophilum*. Pre-treatment with H_2O_2 also affected correlation between gene regulation and pathogen load for several genes. A delay in antibody production in H_2O_2-treated fish in the early phase of infection was indicated, but H_2O_2 exposure did not affect antibody levels 50 days post-infection. An increasing amount of *F. psychrophilum* 16S rRNA was found in the head kidneys of infected fish pre-treated with H_2O_2 relative to the *F. psychrophilum* group. The results show that a single pre-treatment with H_2O_2 impairs the response against *F. psychrophilum* and may intensify infection.

Keywords: Flavobacterium psychrophilum, gene expression, head kidney, hydrogen peroxide, immersion challenge, rainbow trout fry syndrome.

Introduction

The Gram-negative fish pathogen *Flavobacterium psychrophilum* is the causative agent of bacterial cold water disease, also known as rainbow trout fry syndrome (RTFS). With mortality rates ranging up to 70% in fry (Lorenzen *et al.* 1991), the disease continues to have a considerable economic impact on farmed salmonids worldwide. To our knowledge, no commercial vaccine is currently available and the disease is presently treated with antibiotics. Immunoprophylactic strategies may be able to prevent RTFS, but more knowledge is required regarding the immune response in rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *F. psychrophilum* to achieve this goal. Studies carried out so far have relied on the immune response of either naturally infected or injection-challenged fish. Furthermore, focus has been on fish weighing 10–50 g (Overturf & LaPatra 2006; Villarroel *et al.* 2008; Evenhuis & Cleveland 2012; Orieux *et al.* 2013), whereas studies on rainbow trout fry and their response to *F. psychrophilum* are lacking. Injection-based experimental challenges with *F. psychrophilum* are standardized (Madsen & Dalsgaard

Correspondence *M M M Henriksen, Veterinærinstituttet, Sektion for Bakteriologi, Patologi og Parasitologi, Bülowsvej 27, 1870 Frederiksberg C, Denmark (e-mail: mmah@vet.dtu.dk)*

Journal of Fish Diseases

1999), but this route of infection does not reflect a natural situation. Using an experimental immersion challenge, the pathogen will enter the host via surfaces, mimicking a more natural transmission. In the present study, the immune response was investigated using an immersion-based model using hydrogen peroxide (H_2O_2) as pre-treatment (Henriksen, Madsen & Dalsgaard 2013). The model included two infected groups: one was exposed to the bacteria directly, while the other was treated with H_2O_2 prior to pathogen exposure. The challenge method has previously been tested (Henriksen *et al.* 2013), and it has been shown that pre-treatment with H_2O_2 resulted in an increased mortality (9.1–19.2%) from subsequent infection with *F. psychrophilum*. The twofold increase in mortality (14.7–30.3%) was validated in a second experiment (Henriksen *et al.* 2013). Due to the limited existing knowledge regarding the immune response to *F. psychrophilum*, we wished to elucidate both the fast-acting and less specific defence mechanisms of the innate immune response and the adaptive response with a higher degree of specificity and memory. To describe the systemic effects, samples were taken from the head kidney, which is considered to be the central immune organ in fish. Genes encoding the following molecules were investigated: IgT, IgM, CD8, CD4, MHC I, MHC II, IL-4/13A and TcR-β, which are all related to adaptive immunity; IL-10, IL-6, IL-1β, IL-17 and SAA, which are related to inflammation and innate immunity; and finally FoxP3, which is connected to regulatory T cells in mammals, although this link has not yet been established in fish. The expression of immune-relevant genes the first 8 days after immersion in *F. psychrophilum* with or without preceding H_2O_2 immersion was investigated. Bacterial load was assessed by the presence of 16S rRNA and correlated with gene expression. Furthermore, ELISA was used to detect antibody formation 50 days post-infection to reflect development of long-term adaptive immunity.

Materials and methods

Fish

Outbred rainbow trout fry weighing 1.2 g (SD: 0.5) were used for challenge and sampling. The eggs originated from Fousing Trout Farm (Denmark), while disinfection, hatching and initial rearing were carried out at AquaBaltic Hatchery (Bornholm, Denmark). The fish were fed commercial pellets (INICIO Plus, BioMar) and acclimatized for 3 weeks in 200-L tanks with 15 °C recirculated non-chlorinated tap water. The fish were disease-free and confirmed to be free of *F. psychrophilum* upon arrival at the experimental facility as previously described (Henriksen *et al.* 2013). Before randomly selecting the experimental groups, the largest and smallest fish (<0.6 g and >1.5 g) were removed to minimize variation. All tanks, except the tanks used for challenge and H_2O_2 pre-treatment, were opaque in order to minimize external stress due to activity in the experimental facility (Henriksen *et al.* 2013). The work presented in this paper was carried out in agreement with the internationally accepted guidelines for the care and use of laboratory animals in research and under licence (J.nr. 2006/561-1204) issued by the Animal Experiments Inspectorate, Ministry of Justice, Copenhagen, Denmark.

Bacterial strain

Flavobacterium psychrophilum strain 950106-1/1 isolated from a clinical outbreak of RTFS in a Danish freshwater farm in 1995 was used for challenge. Bacterial stock was stored at −80 °C in 15–20% glycerol and pre-cultivated in 10 mL TYES broth at 15 °C for 3 days (110 rpm). Then, 100 mL TYES broth was inoculated using 0.5 mL of the pre-culture and further cultivated for 48 h. The cultures were examined under microscope to verify the purity. Furthermore, colony-forming units were counted following spreading of 0.1 mL in 10-fold dilutions (10^{-4}–10^{-7} in duplicates) on TYES agar (Henriksen *et al.* 2013).

Challenge

A previously published model (Henriksen *et al.* 2013) was used, and the experimental set-up consisted of 4 groups in duplicate with 45 fish in each ($n = 360$): (i) untreated control, (ii) H_2O_2 control, (iii) *F. psychrophilum* exposure and (iv) H_2O_2 pre-treatment followed by *F. psychrophilum* exposure. Treatment with H_2O_2 was performed by immersing the fish in 150 mg L^{-1} H_2O_2 for 60 min. Fish were immediately transferred to another tank containing a 1:9 diluted *F. psychrophilum* broth culture (10^7 CFU mL^{-1}), in which they were immersed for 30 min.

Water temperature during the experiment was 16.8 ± 0.5 °C.

Sampling

For qPCR, 10 fish were sampled from each group (5 from each replicate) before immersion challenge (0 h). Similarly, 10 fish were sampled from each group 4, 48, 125 and 192 h post-challenge. The fish were killed using MS-222 (Sigma-Aldrich, approx. 300 mg L^{-1} stock solution), after which the head kidney was removed aseptically and placed directly in RNA*later* (Sigma-Aldrich). Samples were pre-stored at 4 °C for 48 h and stored at −20 °C until RNA purification took place. Blood samples for the enzyme-linked immunosorbent assay (ELISA) were taken 50 days after challenge from at least 10 fish from each group (5 from each replicate) using heparinized capillary tubes. The tubes were kept in an ice-box while sampling and subsequently centrifuged at 4000 ***g*** for 15 min (Hettich Mikro 200R), before storing plasma at −80 °C.

RNA isolation

All steps in RNA purification were carried out using gloves and filtered tips. Tissues were homogenized using 5-mm stainless steel beads (Qiagen) with TissueLyzer II (Qiagen). Total RNA was isolated using the Maxwell® 16 LEV simplyRNA Tissue Kit by following the manufacturer's instructions. DNase treatment was integrated in the procedure to remove genomic DNA. RNA quality and quantity were determined spectrophotometrically by measuring the optical density at 260/280 nm (NanoDrop 2000c, Thermo Scientific). Subsequently, the RNA concentration was adjusted to the same level in each sample by adding nuclease-free water. RNA was stored at −80 °C until cDNA synthesis.

cDNA synthesis

Synthesis of cDNA was carried out using TaqMan® Reverse Transcription reagents combined with random Oligo(dT) primers (TaqMan® Reverse Transcription, Applied Biosystems), following the instructions provided by the manufacturer. The volume of each reaction was 20 μL, each reaction containing 2.0 μL 10× RT-buffer, 4.4 μL 25 mM $MgCl_2$, 4.0 μL 50 μM dNTP mix, 1.0 μL RNase inhibitor, 1.0 μL reverse transcriptase and 400 ng RNA template. Reverse-transcriptase controls were included. Amplifications were carried out using a Biometra T- 3000 thermocycler amplifier (Biometra). Cycle conditions were 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min.

The resulting cDNA was diluted 10 times in nuclease-free water and stored at −20 °C until use in qPCR.

Real-time PCR: TaqMan® and SYBR green

All real-time quantitative PCR assays were conducted using the same Stratagene MX3005P™ real-time thermocycler (AH Diagnostics as). The synthesized cDNA was used as template in 96-well microplates (AH Diagnostics as), and replicates belonging to the same groups were always run on the same plate. One master mix was used for the analysis of each gene. TaqMan® primers and probes are provided in Table 1. Reaction volumes were 12.5 μL, each reaction containing 2.25 μL nuclease-free H_2O, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL TaqMan probe (5 μM) and 6.25 μL Brilliant II QPCR Master Mix (AH Diagnostics as). Negative controls from the cDNA synthesis were included on each plate, and Elongation Factor 1 (ELF 1-α) was used as reference gene (Raida & Buchmann 2008; Ingerslev *et al.* 2006; Olsvik *et al.* 2005). Initial denaturation at 95 °C for 15 min was followed by 40 cycles, each consisting of denaturation at 95 °C for 30 sec, followed by a combined annealing and elongation step at 60 °C for 30 sec. A SYBR Green qPCR assay was used for quantification of *F. psychrophilum* using previously published primers and probes (Orieux *et al.* 2011). Reaction volumes were 25 μL, each reaction containing 12.5 μL of SYBR Green JumpStart™ Taq ReadyMix™ for Quantitative PCR (Sigma-Aldrich), 2 μM of each primer and nuclease-free water. Cycling conditions were initial denaturation at 96 °C for 10 min, followed by 40 cycles, each consisting of denaturation at 96 °C for 30 s, annealing at 56 °C for 30 s and undergoing primer extension on the DNA template at 72 °C for 30 s. Cut-off was set after 31 cycles, corresponding to approximately 10 CFU, according to the standard curve. This was 1 cycle above the point where not all positive controls were detected, and at least 2 cycles before unspecific replication occurred in uninfected controls. Compared with the paper originally describing the assay

Table 1 Probes and primers used in the study

Gene		Sequence	Amplicon (bp)	Accession no.
EF-1α	Fwd	ACCCTCCTCTTGGTCGTTTC	63	\AF498320
	Rev	TGATGACACCAACAGCAACA		
	Probe	GCTGTGCGTGACATGAGGCA		
IL-10	Fwd	CGACTTTAAATCTCCCATCGAC	70	\AB118099
	Rev	GCATTGGACGATCTCTTTCTTC		
	Probe	CATCGGAAACATCTTCCACGAGCT		
IL-6	Fwd	ACTCCCCTCTGTCACACACC	91	\DQ866150
	Rev	GGCAGACAGGTCCTCCACTA		
	Probe	CCACTGTGCTGATAGGGCTGG		
IL-1β	Fwd	ACATTGCCAACCTCATCATCG	91	\AJ223954
	Rev	TTGAGCAGGTCCTTGTCCTTG		
	Probe	CATGGAGAGGTTAAAGGGTGGC		
SAA	Fwd	GGGAGATGATTCAGGGTTCCA	79	\AM422446
	Rev	TTACGTCCCCAGTGGTTAGC		
	Probe	TCGAGGACACGAGGACTCAGCA		
IL-17c1	Fwd	CTGGCGGTACAGCATCGATA	138	\FM955453
	Rev	GAGTTATATCCATAATCTTCGTATTCGGC		
	Probe	CGTGATGTCCGTGCCCTTTGACGATG		
IL-17c2	Fwd	CTGGCGGTACAGCATCGATA	134	\FM955454
	Rev	CAGAGTTATATGCATGATGTTGGGC		
	Probe	CGTGGTGTCCAGGCCCTTTAATGATG		
FoxP3a	Fwd	CTACAGGCACAGCCTGTCACTAGG	80	\FM883710
	Rev	GCTCCTCTGGCTCTTTAGTGG		
	Probe	CCAGAACCGAGGTGGAGTGTCACG		
FoxP3b	Fwd	TCCTGCCCCAGTACTCATCCC	75	\FM883711
	Rev	GCTCCTCTGGCTCTTTAGTGG		
	Probe	CTTGGCAGCAGATGGAGTGCCACG		
IgT	Fwd	AGCACCAGGGTGAAACCA	73	\AY870265
	Rev	GCGGTGGGTTCAGAGTCA		
	Probe	AGCAAGACGACCTCCAAAACAGAAC		
IgM	Fwd	CTTGGCTTGTTGACGATGAG	72	\S63348
	Rev	GGCTAGTGGTGTTGAATTGG		
	Probe	TGGAGAGAACGAGCAGTTCAGCA		
CD4	Fwd	CATTAGCCTGGGTGGTCAAT	89	\AY973028
	Rev	CCCTTTCTTTGACAGGGAGA		
	Probe	CAGAAGAGAGAGCTGGATGTCTCCG		
CD8	Fwd	ACACCAATGACCACAACCATAGAG	74	\AF178054
	Rev	GGGTCCACCTTTCCCACTTT		
	Probe	ACCAGCTCTACAACTGCCAAGTCGTGC		
MHC I	Fwd	TCCCTCCCTCAGTGTCT	73	\AY523661
	Rev	GGGTAGAAACCTGTAGCGTG		
	Probe	CAGAAGACCCCCTCCTCTCCAGT		
MHC II	Fwd	TGCCATGCTGATGTGCAG	68	\AF115533
	Rev	GTCCCTCAGCCAGGTCACT		
	Probe	CGCCTATGACTTCTACCCCAAACAAAT		
IL-4/13A	Fwd	ATCCTTCTCCTCTCTGTTGC	139	\AB574337
	Rev	GAGTGTGTGTGTATTGTCCTG		
	Probe	CGCACCGGCAGCATAGAAGT		
TcR-β	Fwd	TCACCAGCAGACTGAGAGTCC	73	\AF329700
	Rev	AAGCTGACAATGCAGGTGAATC		
	Probe	CCAATGAATGGCACAAACCAGAGAA		

(Orieux *et al.* 2011), the sensitivity in the present study was lower, which may be related to differences in RNA purification methods and reagents.

All qPCR assays were tested with SYBR Green, and subsequent melting curve analysis was performed to confirm specificity and absence of primer–dimer formation. The amplification efficiency was in all cases within 100% ± 5%.

ELISA

TYES broth was inoculated with *F. psychrophilum* strain 950106-1/1 and grown as described in section 2.2. The culture was centrifuged at 4000 ***g*** for 20 min and washed with PBS twice. The bacterial pellet was sonicated by 15 cycles of 60 s sonication and 30 s pause (Soniprep 150, MSE). Protein content was determined (BCA Protein

Assay Reagent, Pierce Protein Research Products) and diluted to 5 μg protein mL^{-1} in carbonate–bicarbonate buffer (Sigma-Aldrich). Each well on 96-well plates (MaxiSorp, Thermo Fisher Scientific) was coated with 100 μL *F. psychrophilum* sonicate, covered with a plate sealer (Pekema Bar-Com) and incubated overnight at 4 °C. Wells were washed three times with 250 μL wash buffer (PBS (pH 7.2)/0.1% Tween-20 (Sigma-Aldrich) and incubated for 1 h at room temperature with 150 μL blocking buffer (PBS/0.5% bovine serum albumin (BSA/1% Tween-20). Plates were washed three times with wash buffer and stored at −20 °C for later use. Plasma samples were diluted from 1:50 to 1:4000 in assay diluent 5× (PBS/1% BSA/1% Tween-20), and 50 μL was loaded in duplicates and incubated overnight at 4 °C. The plates were washed three times in wash buffer and incubated for 1 h at room temperature with 100 μL 1:500 diluted mouse anti-salmon Ig (MCA2182, AbD Serotec) at 100 rpm. Plates were washed three times and incubated for 1 h at room temperature with 100 μL 1:500 rabbit anti-mouse IgG (STAR13B, AbD Serotec) at 100 rpm. Wells were washed three times using wash buffer and incubated for 10 min at room temperature with 100 μL TMB substrate (Sigma-Aldrich). The reaction was stopped with 100 μL 1 N HCl. Finally, absorbance was measured at 450 nm. Cut-off was determined to a confidence level of 99.9% (Frey, Di Canzio & Zurakowski 1998).

Data analysis of qPCR

Each fish was treated individually throughout data analysis. C_t values were determined as the lowest possible on the linear slopes of plotted log (fluorescence) and C_t values. Data are presented as fold changes in expression and determined according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001), where $\Delta C_t = C_t(\text{target}) - C_t(\text{ELF-1})$ and $\Delta\Delta C_t = \Delta C_t(\text{treatment}) - \Delta C_t(\text{control})$. Subsequently, ΔC_t values from the control group at each time point were compared to the control (0 h), which was taken before any treatment took place and, treated groups were compared to the untreated control at each time point. No significant differences were found throughout the experimental period. Shapiro–Wilk's test was used to ascertain the normal distribution of data, before comparing experimental groups at each time point using Student's *t*-test (1-tail) in Excel. Results were considered to be significant when both $P \leq 0.05$ and at least threefold changes were found. Relative pathogen loads (log10 CFU per 400 ng RNA) for the two infected groups were compared using Student's *t*-test (2-tail) in Excel. Furthermore, gene expression levels (fold changes) were correlated with relative pathogen load (16S RNA transcripts) in the individual groups using Pearson product-moment correlation coefficient. Pathogen load is stated as a relative value, because the concentration of RNA was adjusted to the same level in each sample after purification, rather than using identical amounts of tissue. Results from ELISA were compared using 1-way ANOVA and Tukey's post-test for comparison between groups. GraphPad Prism 5 was used for creating figures.

Results

Quantification of *F. psychrophilum* in the head kidney

The relative *F. psychrophilum* load after 4 h was significantly higher ($P \leq 0.01$) in the group exposed solely to *F. psychrophilum* (Fig. 1) compared with the H_2O_2 + *F. psychrophilum* group; in the *F. psychrophilum* group, 100% of the samples were positive above the limit of detection compared with 80% in the H_2O_2 + *F. psychrophilum* group. After 48 h, the pathogen level had decreased in both groups, but with a tendency towards an increase in the H_2O_2 + *F. psychrophilum* group. After 192 h, a significantly higher number of bacteria were found in the group exposed to both H_2O_2 and *F. psychrophilum* ($P \leq 0.05$); in the *F. psychrophilum* group, 20% of the samples were positive compared with 50% in the H_2O_2 + *F. psychrophilum* group. No positive fish were recorded in any of the control groups.

qPCR gene expression

The constitutive expression of each gene is shown in Table 2, while fold changes in gene expression over time are summarized in Table 3, and correlations between pathogen load and immune gene expression are shown in Table 4. Significant changes in regulations are stated below.

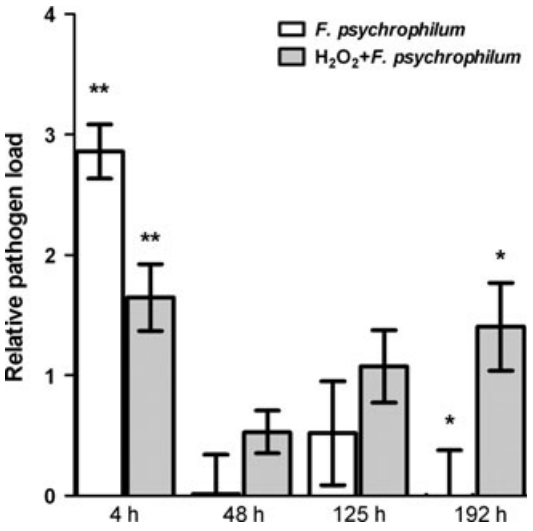


Figure 1 Relative pathogen levels measured by qPCR. The relative pathogen load shown as log10 CFU over time. Significance levels are denoted with asterisks ($^{*}P < 0.05$ and $^{**}P < 0.001$).

Table 2 Constitutive gene expression before treatment. Real-time cycle threshold (Ct) values along with SEM are given for each gene

Gene	C_t	SEM
EF1-α	20.36	0.55
IL-10	31.44	0.55
IL-6	36.70	0.99
IL-1β	32.16	0.83
SAA	25.88	1.24
IL-17c1	37.53	1.34
IL-17c2	34.08	0.60
FoxP3a	32.29	0.45
FoxP3b	31.21	0.67
IgT	28.81	0.57
IgM	21.70	0.57
CD8	28.18	0.63
CD4	31.75	0.97
MHC I	26.34	0.60
MHC II	24.70	0.77
IL-4/13A	30.42	0.75
TcR-β	25.86	0.55

After 4 h, a fivefold down-regulation was seen for IgT in the H_2O_2 + *F. psychrophilum* group, while a three- to four-fold up-regulation was seen for MHC I and IL-1β in both infected groups (Table 3). A positive correlation was found between *F. psychrophilum* load and gene expression in the *F. psychrophilum* group with regard to FoxP3a, IgT and IgM (Table 4).

After 48 h, a 10-fold down-regulation of IL-17c1 was seen in the H_2O_2 + *F. psychrophilum* group, while IL-10 was down-regulated three- to four-fold in all groups, including the H_2O_2-treated group. No correlation was found between regulation of gene expression and bacterial load.

After 125 h, IgT was down-regulated nine-fold in the *F. psychrophilum* group, and MHC II was down-regulated 3- to 4-fold in both infected groups. In the *F. psychrophilum* group, a positive correlation was found between bacterial load and IL-17c1, SAA, CD4 and MHC I, while only SAA correlated in the H_2O_2 + *F. psychrophilum* group.

After 192 h, FoxP3a was down-regulated five-fold and FoxP3b down eight-fold in the *F. psychrophilum* group and the latter gene was down-regulated four-fold in the H_2O_2 + *F. psychrophilum* group. IL-17c1 was down-regulated 12-fold in the uninfected H_2O_2-treated group and eight- to nine-fold in both infected groups. IL-10 was down-regulated nine-fold in the uninfected H_2O_2-treated group, while a down-regulation of five- to six-fold was seen in both infected groups. IL4/13 was down-regulated three-fold in the uninfected H_2O_2-treated group and five-fold in the *F. psychrophilum* group. TcR-β was down-regulated three-fold in the *F. psychrophilum* group, and IL-1β was down-regulated four-fold in both the H_2O_2-treated group and *F. psychrophilum* group. A negative correlation was found between bacterial load and regulation of IL-17c1 in the *F. psychrophilum* group, while regulation of IL-10 and IgM was positively correlated in the H_2O_2 + *F. psychrophilum* group.

ELISA

Fifty days after infection, the relative antibody levels were 0 for the two control groups and 3309 (SD: 0.48) and 3072 (SD: 0.37) for the *F. psychrophilum* and H_2O_2 + *F. psychrophilum* groups, respectively. There was no significant difference in the level of *F. psychrophilum*-specific antibodies in the two infected groups (Fig. 2).

Discussion

A few previous studies have investigated the immune response to infection with *Flavobacterium psychrophilum* in fish (Overturf & LaPatra 2006; Villarroel *et al.* 2008; Evenhuis & Cleveland 2012; Orieux *et al.* 2013), but none of those investigations were based on an immersion-based experimental model. As RTFS has the greatest impact on

Table 3 Changes in gene expression 4, 48, 125 and 192 h after exposure in H_2O_2, *F. psychrophilum* (*F.p.*) or both

	4 h						48 h					
	H_2O_2	SEM	*F.p.*	SEM	H_2O_2+ *F.p.*	SEM	H_2O_2	SEM	*F.p.*	SEM	H_2O_2+ *F.p.*	SEM
IL-10	1.36	0.47	1.11	0.56	1.73	0.69	**−3.54***	0.72	**−3.29****	0.33	**−3.56****	0.43
IL-6	−1.18	1.46	1.22	0.78	−2.18	0.21	−2.06	0.43	−1.56	0.55	−1.65	0.73
IL-1β	1.46	0.41	**3.66***	0.56	**3.03***	0.48	−1.13	0.57	1.61	0.30	−1.06	0.39
SAA	1.18	0.30	1.38	0.50	1.27	0.63	−2.88	0.41	−1.37	0.41	−1.66	0.50
IL-17c1	−1.74	0.63	−1.30	0.94	1.01	0.94	−2.25	0.77	−1.37	0.94	**−10.13***	1.28
IL-17c2	1.90	0.84	1.81	0.55	2.63	1.90	−1.11	0.87	1.27	0.47	−1.09	0.72
FoxP3a	1.78	0.26	1.33	0.55	1.86	0.48	1.13	0.27	1.02	0.22	−1.63	0.39
FoxP3b	1.61	0.28	2.33	0.48	1.91	0.56	−1.07	0.32	1.10	0.26	−1.48	0.41
IgT	1.63	0.74	−1.52	1.07	**−5.25****	0.86	1.10	0.54	1.32	0.82	−1.47	0.85
IgM	−1.62	0.62	−1.39	0.39	−1.30	0.52	−2.25	0.44	−2.12	0.57	−1.18	0.49
CD8	1.25	0.23	1.33	0.32	−1.57	0.84	1.58	0.31	1.97	0.48	1.01	0.46
CD4	1.89	0.73	3.07	0.67	1.26	0.40	1.54	0.44	2.61	0.50	2.10	0.55
MHC I	1.89	0.49	**3.20***	0.41	**3.86****	0.25	−1.35	0.38	1.49	0.33	−1.16	0.29
MHC II	−1.04	0.36	−1.09	0.57	−1.94	0.31	1.02	0.24	1.58	0.44	−1.11	0.34
IL-4/13A	−1.43	0.34	1.01	0.47	1.03	0.36	−1.17	0.29	−1.25	0.24	−1.81	0.20
TcR-β	1.16	0.35	1.67	0.35	−1.09	0.42	1.17	0.34	1.69	0.39	1.14	0.44
	125 h						192 h					
IL-10	1.94	0.56	−1.42	0.66	−1.85	0.62	**−9.06*****	0.41	**−5.68****	0.52	**−5.35***	0.75
IL-6	−1.22	0.47	1.13	0.74	2.78	0.66	−3.75	0.76	−1.51	1.05	−6.51	1.45
IL-1β	1.20	0.35	−1.12	0.30	1.03	0.47	**−4.11****	0.54	**−3.87****	0.35	−2.43	0.54
SAA	−1.02	0.27	1.89	0.81	2.85	0.73	−1.66	0.98	1.57	1.39	−1.52	2.48
IL-17c1	−2.10	0.80	−2.58	0.67	−1.85	0.88	**−12.18*****	0.75	**−8.65*****	0.60	**−7.82****	0.81
IL-17c2	−1.87	0.44	−2.21	0.74	−2.66	1.01	−1.36	0.80	−1.23	0.34	1.20	1.21
FoxP3a	1.75	0.47	1.07	0.40	−1.58	0.46	−2.24	0.41	**−5.31****	0.37	−2.82	0.61
FoxP3b	1.87	0.47	−1.03	0.42	−2.10	0.50	−4.13	0.47	**−8.03*****	0.44	**−3.73****	0.49
IgT	−5.68	1.07	**−8.70****	0.89	−2.36	0.53	−5.33	1.79	−2.45	0.74	1.98	0.59
IgM	−2.41	1.11	−1.30	0.50	−1.02	0.52	−2.86	0.76	−2.30	0.53	−1.37	0.30
CD8	−1.54	0.38	−1.27	0.49	−1.30	0.25	2.23	0.91	−2.16	1.23	−2.90	2.37
CD4	1.90	0.42	1.20	0.62	1.30	0.49	1.47	0.69	−1.95	0.63	2.98	0.54
MHC I	1.58	0.80	2.91	0.55	2.65	0.28	−1.33	0.47	−1.27	0.39	1.54	0.62
MHC II	−2.48	0.63	**−3.33****	0.35	**−3.54****	0.43	−1.29	0.35	−2.27	0.48	1.43	0.39
IL-4/13A	−1.07	0.38	−1.28	0.54	−2.01	0.39	**−3.14***	0.46	**−5.27*****	0.37	−2.45	0.43
TcR-β	1.62	0.36	1.56	0.34	−1.08	0.32	−2.17	0.66	**−2.99****	0.39	−1.80	0.47

Changes are given as mean changes in fold with standard error (SEM), and significance levels are denoted with asterisks (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). Bold numbers are statistically significant and negative values indicate a down-regulation, while positive values indicate up-regulations.

fry, rainbow trout weighing approximately 1.2 g were used in the present study, which has been shown to be above the lower limit for activation of adaptive immunity and development of protective immunity (Buchmann, Nielsen & Nielsen 2003; Chettri *et al.* 2012). Two infected groups were analysed: one group was immersed directly into a diluted bacterial broth, while the other was pre-treated with H_2O_2 before immersion. Exposure to a sufficient concentration of almost any chemical or contaminant induces a stress response (Harper & Wolf 2009). H_2O_2 has been shown to accelerate and elevate mortality of *Tenacibaculum maritimum* infections in turbot (Avendaño-Herrera *et al.* 2006), *Flavobacterium columnare* infections in channel catfish (Thomas-Jinu & Goodwin 2004) and *F. psychrophilum* infections in rainbow trout (Henriksen *et al.* 2013); in the last case, pre-treatment with H_2O_2 roughly doubled the percentage point mortality. Furthermore, single treatments with H_2O_2 have been shown to have a short-term stress effect on Atlantic salmon, *Salmo salar* L., resulting in elevated concentrations of glucose and cortisol in the plasma (Bowers, Speare & Burka 2002). Another study showed that damage due to H_2O_2 primarily occurred in the gills and that pathological changes can include oedemas, lamellar fusion, epithelial hyperplasia as well as swelling and lifting of the gill epithelium (Tort *et al.* 2002). Hence, a disruption of the epidermal integrity cannot be excluded and may also lead to an increased vulnerability to pathogens. Generally, considerable individual variation in regulation of genes complicated interpretation, an issue that has been

Table 4 Correlation of gene expression and pathogen load in the two infected groups; *F. psychrophilum* alone ($-H_2O_2$) or pre-treatment with H_2O_2 before *F. psychrophilum* exposure ($+H_2O_2$)

	4 h		48 h		125 h		192 h	
	$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$	$-H_2O_2$	$+H_2O_2$
IL-10								0.84*
IL-6								
IL-1β								
SAA					0.96**	0.94*		
IL-17c1					0.89*		−0.98*	
IL-17c2								
FoxP3a	0.68*							
FoxP3b								
IgT	0.87*							
IgM	0.85*							0.81*
CD4					0.91*			
CD8								
MHC I					0.86*			
MHC II								
IL-4/13A								
TcR-β								

Positive numbers signify a positive correlation and negative numbers a negative correlation. Pearson's r is given for all significant associations. Significance levels are denoted by the number of characters (*$P < 0.05$ and **$P < 0.01$).

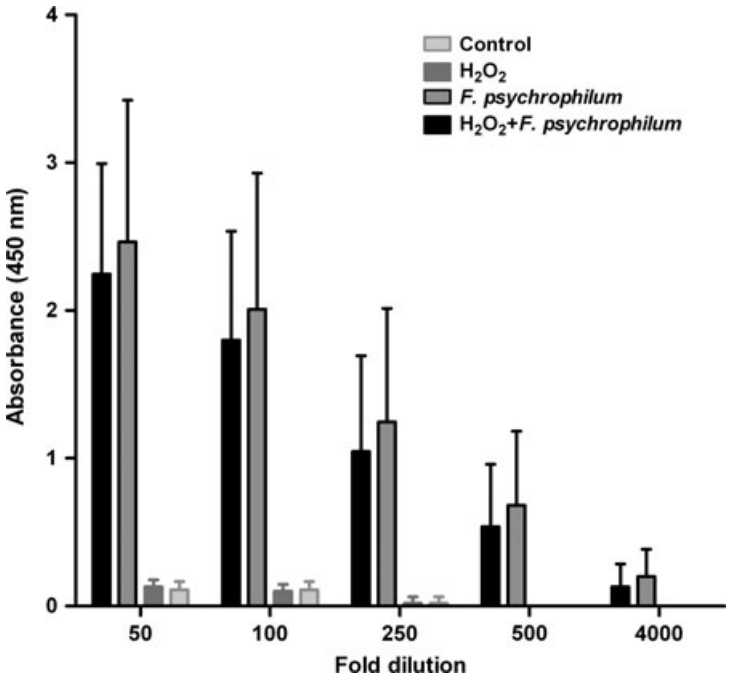


Figure 2 Antibody levels 50 days post-infection.

previously recognized by Overturf & LaPatra (2006). Overall, minor differences were seen for the measured immune parameters. Therefore, we have included a correlation analysis of pathogen load and gene expression in the present work.

SAA is an acute-phase protein, primarily produced in the liver. It binds to invading pathogens and may recruit leucocytes in the early phases of an inflammatory response (Kania, Chettri & Buchmann 2013). In the current work, no significant regulations were found in any of the two infected groups. However, SAA transcription positively correlated with pathogen load in both groups 125 h after infection. This can, at least partly, be explained by choice of tissue, size of the fish and challenge method. Additionally, the SAA

gene was subject to high individual variation, both for constitutive expression in untreated fish and after 125 and 192 h in infected fish. A previous study focused exclusively on the expression of SAA in rainbow trout and found an up-regulation of the gene in intestine, skin, liver and gills of fish naturally infected with *F. psychrophilum* (Villarroel *et al.* 2008). This suggests participation in local response to damage, and the onset of inflammation. Furthermore, the SAA content in plasma did not increase, which suggests a role for SAA in the local inflammatory response.

IL-10 is an anti-inflammatory regulatory cytokine affecting the expression of other inflammatory cytokines, such as IL-6 and IL-1β. IL-10 has several other biological effects and is associated with macrophages. Up-regulation of IL-10 has previously been noted in rainbow trout after exposure to *Y. ruckeri*, indicating participation in the immune response after infection (Raida & Buchmann 2007). In the present study, an up-regulation of IL-1β and down-regulation of IL-10 was observed in both infected groups. Overall, a pro-inflammatory response was seen, although the positive correlation between IL-10 regulation and pathogen load in the H_2O_2 + *F. psychrophilum* group 192 h post-challenge could be interpreted as a part of a suppressive effect on the immune response elicited by *F. psychrophilum*. The effect may also be caused by the release of cortisol as a response to stress, because cortisol is known to reduce inflammation and suppress the immune system. Immersion-based experimental infections with *F. psychrophilum* have resulted in little or no mortality without the application of stress or scarification (Nematollahi *et al.* 2003; Henriksen *et al.* 2013); a delay or reduction in the inflammatory response could give the pathogen better opportunity to overcome the host's immune system. Inflammation is a balance between pro- and anti-inflammatory cytokines, and regulation is necessary to reduce the amount of self-damage caused by the immune response. In mammals, IL-6 is a pleiotropic cytokine regulating haematopoiesis, inflammation, immune responses and bone homeostasis and may well have the same effect in rainbow trout (Costa *et al.* 2011). However, it is noteworthy that no changes in IL-6 expression were observed in the present study. IL-1β is a pro-inflammatory cytokine in mammals and is also believed to be an important player in the instigation of early immune responses in rainbow trout. IL-1β has previously been linked to adhesion, colonization and invasion of bacterial pathogens (Komatsu *et al.* 2009) and was shown to be up-regulated in gills, spleen, liver and head kidney of farmed rainbow trout naturally infected with RTFS (Orieux *et al.* 2013).

FoxP3 is a transcriptional factor expressed by naturally occurring regulatory T cells in mammals. Regulatory T cells suppress other leucocytes to maintain self-tolerance and immune homeostasis (Sakaguchi *et al.* 2009). Two paralogue genes, FoxP3a and FoxP3b, have been identified in rainbow trout, and although their functions are still largely unknown, expression is subject to differential modulation and may prove to have different functions (Wang *et al.* 2010b). In the present study, both FoxP3a and FoxP3b genes were down-regulated in the *F. psychrophilum* group, while only FoxP3b was down-regulated in the H_2O_2 + *F. psychrophilum* group; a positive correlation with pathogen load was found early after exposure in the *F. psychrophilum* group only. This suggests that *F. psychrophilum* does not induce any tolerance-associated reactions.

IL-17 plays an important role in mammals for the stimulation of pro-inflammatory reactions, including recruitment of neutrophil cells. Two homologue genes have been discovered in rainbow trout: IL-17c1 and IL-17c2 (Wang *et al.* 2010a). In rainbow trout weighing around 100 g, IL-17c1 was mainly expressed in gills and skin, while IL-17c2 was expressed in spleen, head kidney and brain. However, the effect of IL-17 is still unexplored in rainbow trout. Expressions of both genes were higher in mucosal surfaces and were significantly up-regulated after injection with *Y. ruckeri* (Wang *et al.* 2010a). In the present study, no regulatory changes in IL-17c2 were observed, while down-regulation of IL17c1 was fastest in the H_2O_2 + *F. psychrophilum* group. The comparatively strong down-regulation detected in the H_2O_2 + *F. psychrophilum* group suggests a suppressive effect on the immune system elicited by *F. psychrophilum*.

CD4 and CD8 are co-receptors facilitating communication between the T-cell receptor (TcR) and an antigen-presenting cell in mammals, and regulation of the TcR-β-chain is an indirect measure for the presence of T cells in the tissue. CD4-positive T cells recognize extracellularly derived antigens presented by MHC II molecules and differentiate into either T_h1 effector cells

activating macrophages or T_h2 cells stimulating proliferation of antibody producing B-cells. CD8-positive T cells recognize intracellularly derived antigens which are presented by MHC I molecules. The CD8-positive cells recognizing MHC I differentiate into cytotoxic T cells. Previously, expression of CD8 and MHC II has been shown to be repressed in rainbow trout naturally infected with *F. psychrophilum* (Orieux *et al.* 2013), while an up-regulation of CD8 has been observed in the spleen of injection-challenged fish (Overturf & LaPatra 2006). In the present study, no significant changes in the regulation of gene expression for either CD4 or CD8 were found. In both infected groups, an up-regulation of MHC I was observed shortly after infection, while MHC II was down-regulated later on. Both IL4/13A and TcR-β was down-regulated in the *F. psychrophilum* group at the final sampling. Furthermore, a positive correlation was found between pathogen load and regulation of both CD4 and MHC I in the *F. psychrophilum* group. Overall, no clear picture of either a T_h1 or T_h2 response appeared, leaving room for speculation as to the importance of cell-mediated immunity in protection against *F. psychrophilum*.

Several studies have investigated the pathological effects of H_2O_2 on salmonids to establish recommendations for safe application on fish (Arndt & Wagner 1997; Rach *et al.* 1997; Speare & Arsenault 1997; Gaikowski, Rach & Ramsay 1999; Tort *et al.* 2002), because it is used for routine water treatments in aquaculture. The applied H_2O_2 concentration in the present study was within previously established recommended treatment dosages and resulted in 3.5% mortality during treatment (Henriksen *et al.* 2013). Several regulatory changes followed the 60 min of immersion in 150 mg L^{-1} H_2O_2: after 48 h, IL-10 was down-regulated, and after 192 h, genes encoding for IL-10, IL-1β, IL-17c1 and IL4/13A were down-regulated. By comparing the adaptive immune response to *F. psychrophilum* with and without preceding exposure to H_2O_2, the effect on the immune response was evaluated. A down-regulation of IgT and IL-17c1 was observed earlier after infection in the H_2O_2 + *F. psychrophilum* group than in the *F. psychrophilum* group, while down-regulation of FoxP3a, TcR-B and IL4/13A was only found in the *F. psychrophilum* group. When looking at correlation with pathogen load, the positive correlation with IgM was delayed by H_2O_2, while CD4, MHC I and IL-17c1 only positively correlated in the *F. psychrophilum* group. A correlation with pathogen load was also found for IL-17c1 in the *F. psychrophilum* group, while IL-10 was positively correlated in the H_2O_2 + *F. psychrophilum* group after 192 h. Thus, exposure to H_2O_2 influences the regulation of several immune-relevant genes following infection with *F. psychrophilum* and may partly explain the increased mortality in the H_2O_2 + *F. psychrophilum* group (Henriksen *et al.* 2013).

A previous study demonstrated that immersion of rainbow trout fry in live *F. psychrophilum* led to the development of protective immunity to secondary injection-challenge 50 days post-exposure (Lorenzen *et al.* 2010). Rainbow trout fry have also been passively immunized applying sera of larger animals exposed to live bacteria. Another study examined gene expression in the intestine of *F. psychrophilum*-infected fish and demonstrated an up-regulation of IgM 10 days after infection (Evenhuis & Cleveland 2012). This resulted in protection and indicated that antibodies could play a role in partial protection against *F. psychrophilum* (LaFrentz *et al.* 2003). These results were contradicted by a recent study using naturally infected trout, where the authors found no correlation between IgM titre and health status, size or origin of the fish (Orieux *et al.* 2013). In the present study, exposure to H_2O_2 prior to infection did not significantly affect the relative antibody levels in the blood 50 days after infection, although a trend towards higher levels in the *F. psychrophilum* group was observed. The IgT gene was down-regulated earlier in the H_2O_2 + *F. psychrophilum* group, and no regulation of IgM was observed in either of the infected groups. However, a positive correlation between pathogen load and gene expression for both IgT and IgM was found after 4 h in the *F. psychrophilum* group, while only IgM expression correlated to pathogen load in the H_2O_2 + *F. psychrophilum* group and only after 192 h. This suggests a delay in the production of antibodies for the H_2O_2 + *F. psychrophilum* fish in the early phase of infection.

In mature rainbow trout, *F. psychrophilum* cells may be phagocytosed, triggering the production of reactive oxygen species, which kill the pathogen. However, virulent *F. psychrophilum* strains have a higher rate of survival inside macrophages (Nematollahi *et al.* 2005). In the present study, it was found that the pathogen was already present in

the head kidney 4 h after infection. At this early time point, the highest amount was found in the *F. psychrophilum* group, but after 192 h, a significantly higher amount was found in the H_2O_2 + *F. psychrophilum* group.

Indications of a pro-inflammatory response were observed post-infection in both *F. psychrophilum*-infected groups. In the H_2O_2 + *F. psychrophilum* group, however, correlation between pathogen load and gene regulation was scarce compared with the *F. psychrophilum* group. Only a weak coherent pattern of an adaptive response was detected, which was most evident in the *F. psychrophilum* group. The gills and skin are important tissues to examine further using immersion challenge, because they might be damaged by H_2O_2 and serve as portals of entry for the pathogen. Further investigation is also needed to elucidate the possible immunosuppressive actions of *F. psychrophilum* and impact of routine treatments with H_2O_2 on RTFS outbreaks.

Acknowledgements

This work was performed under the Danish Fish Immunology Research Centre and Network (DAFINET) and was supported by the Danish Council of Strategic Research. The technical assistance provided by Lene Gertman during sampling was greatly appreciated.

References

Arndt R.E. & Wagner E.J. (1997) The toxicity of hydrogen peroxide to rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clarki* fry and fingerlings. *Journal of the World Aquaculture Society* **28**, 150–157.

Avendaño-Herrera R., Magariños B., Irgang R. & Toranzo A.E. (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). *Aquaculture* **257**, 104–110.

Bowers J.M., Speare D.J. & Burka J.F. (2002) The effects of hydrogen peroxide on the stress response of Atlantic Salmon (*Salmo salar*). *Journal of Veterinary Pharmacology and Therapeutics* **25**, 311–313.

Buchmann K., Nielsen M.E. & Nielsen C.V. (2003) Immune responses against *Yersinia ruckeri* have no effect on colonization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), by *Gyrodactylus derjavini* (Mikailov, 1975). *Journal of Fish Diseases* **26**, 183–186.

Chettri J.K., Raida M.K., Kania P.W. & Buchmann K. (2012) Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. *Developmental and Comparative Immunology* **36**, 463–474.

Costa M.M., Maehr T., Diaz-Rosales P., Secombes C.J. & Wang T. (2011) Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: effects on macrophage growth and antimicrobial peptide gene expression. *Molecular Immunology* **48**, 1903–1916.

Evenhuis J.P. & Cleveland B.M. (2012) Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. *Veterinary Immunology and Immunopathology* **146**, 8–17.

Frey A., Di Canzio J. & Zurakowski D. (1998) A statistically defined endpoint titer determination method for immunoassays. *Journal of Immunological Methods* **221**, 35–41.

Gaikowski M.P., Rach J.J. & Ramsay R.T. (1999) Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. *Aquaculture* **178**, 191–207.

Harper C. & Wolf J.C. (2009) Morphologic effects of the stress response in fish. *Ilar Journal* **50**, 387–396.

Henriksen M.M.H., Madsen L. & Dalsgaard I. (2013) Effect of hydrogen peroxide on immersion challenge of rainbow trout fry with *Flavobacterium Psychrophilum*. *PLoS ONE* **8** (4).

Ingerslev H.C., Pettersen E.F., Jakobsen R.A., Petersen C.B. & Wergeland H.I. (2006) Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). *Molecular Immunology* **43**, 1194–1201.

Kania P.W., Chettri J.K. & Buchmann K. (2013) Serum Amyloid A: Gene expression and immunohistochemical localization in rainbow trout, *Oncorhynchus mykiss*, infected by *Yersinia ruckeri* In: *DAFINET Workshop and Ph.D. Course: Book of abstracts*, pp. 27. Copenhagen, Denmark.

Komatsu K., Tsutsui S., Hino K., Araki K., Yoshiura Y., Yamamoto A., Nakamura O. & Watanabe T. (2009) Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. *Developmental and Comparative Immunology* **33**, 499–506.

LaFrentz B.R., LaPatra S.E., Jones G.R. & Cain K.D. (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. *Journal of Fish Diseases* **26**, 377–384.

Livak K.J., Schmittgen T.D.. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ Method. *Methods (San Diego, Calif.)* **25**, 402–408.

Lorenzen E., Dalsgaard I., From J., Hansen E.M., Hørlyck V., Korsholm H., Mellergaard S. & Olsen N.J. (1991) Preliminary investigations of fry mortality syndrome in rainbow trout. *Bulletin of the European Association of Fish Pathologists* **11**, 77–79.

Lorenzen E., Brudeseth B.E., Wiklund T. & Lorenzen N. (2010) Immersion exposure of rainbow trout (*Oncorhynchus mykiss*) fry to wildtype *Flavobacterium psychrophilum* induces

no mortality, but protects against later intraperitoneal challenge. *Fish & Shellfish Immunology* **28**, 440–444.

Madsen L. & Dalsgaard I. (1999) Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **36**, 169–176.

Nematollahi A., Decostere A., Pasmans F. & Haesebrouck F. (2003) *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases* **26**, 563–574.

Nematollahi A., Pasmans F., Haesebrouck F. & Decostere A. (2005) Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **64**, 23–28.

Olsvik P.A., Lie K.K., Jordal A.E., Nilsen T.O. & Hordvik I. (2005) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology* **6**, 21.

Orieux N., Bourdineaud J., Douet D., Daniel P. & Le Hénaff M. (2011) Quantification of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), tissues by qPCR. *Journal of Fish Diseases* **34**, 811–821.

Orieux N., Douet D.G., Le Henaff M. & Bourdineaud J.P. (2013) Prevalence of *Flavobacterium psychrophilum* bacterial cells in farmed rainbow trout: Characterization of metallothionein A and interleukin1-beta genes as markers overexpressed in spleen and kidney of diseased fish. *Veterinary Microbiology* **162**, 127–135.

Overturf K. & LaPatra S. (2006) Quantitative expression (Walbaum) of immunological factors in rainbow trout, *Oncorhynchus mykiss* (Walbaum), after infection with either *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, or infectious haematopoietic necrosis virus. *Journal of Fish Diseases* **29**, 215–224.

Rach J.J., Schreier T.M., Howe G.E. & Redman S.D. (1997) Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *Progressive Fish-Culturist* **59**, 41–46.

Raida M.K. & Buchmann K. (2007) Temperature-dependent expression of immune-relevant genes in rainbow trout following *Yersinia ruckeri* vaccination. *Diseases of Aquatic Organisms* **77**, 41–52.

Raida M.K. & Buchmann K. (2008) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. *Fish & Shellfish Immunology* **25**, 533–541.

Sakaguchi S., Wing K., Onishi Y., Prieto-Martin P. & Yamaguchi T. (2009) Regulatory T cells: how do they suppress immune responses? *International Immunology* **21**, 1105–1111.

Speare D.J. & Arsenault G.J. (1997) Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 2653–2658.

Thomas-Jinu S. & Goodwin A.E. (2004) Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. *Journal of Fish Diseases* **27**, 23–28.

Tort M.J., Jennings-Bashore C., Wilson D., Wooster G.A. & Bowser P.R. (2002) Assessing the effects of increasing hydrogen peroxide dosage on rainbow trout gills utilizing a digitized scoring methodology. *Journal of Aquatic Animal Health* **14**, 95–103.

Villarroel F., Casado A., Vásquez J., Matamala E., Araneda B., Amthauer R., Enriquez R. & Concha M.I. (2008) Serum amyloid A: a typical acute-phase reactant in rainbow trout? *Developmental and Comparative Immunology* **32**, 1160–1169.

Wang T., Martin S.A.M. & Secombes C.J. (2010a) Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated. *Developmental & Comparative Immunology* **34**, 491–500.

Wang T., Monte M.M., Huang W., Boudinot P., Martin S.A.M. & Secombes C.J. (2010b) Identification of two FoxP3 genes in rainbow trout (*Oncorhynchus mykiss*) with differential induction patterns. *Molecular Immunology* **47**, 2563–2574.

Received: 23 August 2013
Revision received: 20 September 2013
Accepted: 22 September 2013

PAPER III

Effect of hydrogen peroxide and/or *Flavobacterium psychrophilum* on the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Maya Maria Mihályi Henriksen[a], Per Walter Kania[b], Kurt Buchmann[b] and Inger Dalsgaard[a]

[a]Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute,Technical University of Denmark, Frederiksberg C, Denmark.

[b] Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

Short title: Impact of H_2O_2 and *F. psychrophilum* on the gills

Abstract

The immune response and morphological changes in the gills of rainbow trout fry after immersion in hydrogen peroxide (H_2O_2), *Flavobacterium psychrophilum* or combined exposure were examined. The gills were sampled 4 h, 48 h, 125 h and 192 h after exposure and the regulation of expression of the following genes was investigated using qPCR: IgT, IgM, CD8, CD4, MHC I, MHC II, IL-4/13A, TcR-β, IL-10, IL-1β, IL-17, SAA and FoxP3. Bacteria were not observed in hematoxylin and eosin stained gill tissue, but the presence of *F. psychrophilum* 16S rRNA was detected using qPCR. The 16S rRNA levels were correlated with gene expression. Although pre-treatment with H_2O_2 before immersion in *F. psychrophilum* did not significantly alter the amount of bacteria found in the gill, the immune response was influenced: Exposure to *F. psychrophilum* resulted in a negative correlation with expression of IL-17c1, MHC I and MHC II, while pre-treatment with H_2O_2 resulted in a positive correlation with IL-4/13A and IgM. Exposure to either H_2O_2 or *F. psychrophilum* influenced regulation of gene expression and damaged tissue. Exposure to both combined altered the immune response to infection and postponed healing of gill tissue.

Keywords: *Flavobacterium psychrophilum*, rainbow trout fry syndrome, hydrogen peroxide, immersion challenge, gills, gene expression

1. Introduction

Flavobacterium psychrophilum is a Gram negative bacterium and the cause of rainbow trout fry syndrome (RTFS) in farmed rainbow trout, *Oncorhynchus mykiss* (Lorenzen *et al.* 1991). *F. psychrophilum* has been demonstrated in both skin, connective tissue of the fins, gills and operculum of salmonid fish, suggesting it is a part of the normal microflora on skin and gills (Lorenzen 1994; Holt, Rohovec & Fryer 1993; Madetoja, Dalsgaard & Wiklund 2002). If fish are immune-suppressed due to environmental conditions, *F. psychrophilum* is thought to be able to gain entry through skin and gills (Nematollahi, Decostere, Pasmans & Haesebrouck 2003). The gills are considered to be an important organ for entry and rapid distribution of a number of pathogens, such as the bacterial fish pathogen *Yersinia ruckeri* (Tobback *et al.* 2009), although the specific ports of entry are still disputed (Khimmakthong *et al.* 2013; Deshmukh *et al.* 2013). Recent research suggests that the gills have a role in infection with *Aeromonas salmonicida* and *Vibrio anguillarum* as well (Jayasuriya *et al.* 2013; Kato, Takano, Sakai, Matsuyama & Nakayasu 2013). Highly virulent strains of *F. columnare* have been demonstrated to destroy the gills and form large clusters resembling biofilm in carp, *Cyprinus carpio* (Declercq *et al.* 2013). *F. branchiophilum* is the causative agent of bacterial gill disease and produce characteristic aggregations in the gills under poor environmental conditions (Good, Thorburn & Stevenson 2008). Furthermore, a positive correlation between the adhesion capacity to the gills and virulence of *F. psychrophilum* has been demonstrated (Nematollahi, Decostere, Pasmans, Ducatelle *et al.* 2003). The gills are vulnerable to both physical and chemical damage due to location and function. Contact with non-medical compounds, such as hydrogen peroxide (H_2O_2), can result in increased mortality rates from subsequent exposure to pathogens (Henriksen, Madsen & Dalsgaard 2013; Avendaño-Herrera, Magariños, Irgang & Toranzo 2006; Thomas-Jinu & Goodwin 2004). Mucosal surfaces are considered to be important entry-points for pathogens, since they function as permeable barriers in contact with the environment (Khimmakthong *et al.* 2013). Injection-based challenge is suboptimal for investigations regarding potential portals of entry and the local immune response, since the mucosal surfaces, such as the skin, intestine and gills, are circumvented.

The aim of the present study was to gain knowledge regarding how exposure to *F. psychrophilum* and H_2O_2 affects immunity and morphology in the gills. Rainbow trout fry were immersed in *F. psychrophilum* with and without H_2O_2 pre-treatment (Henriksen, Madsen *et al.* 2013), and samples were taken from the gills during the first eight days post exposure. The regulation of several genes related primarily to adaptive immunity was examined by qPCR (IgM, IgT, MHC I, MHC II, CD4,

CD8, IL-4/13A, TcR-β, IL-10, IL-6, IL-1β, IL-17, FoxP3, SAA). Also, the relative pathogen-load in the gills was assessed. Bacteriological samples were taken from liver, kidney and spleen of animals dying from RTFS to determine whether H_2O_2 pre-treatment affected the distribution of *F. psychrophilum*. Finally, gross histological changes in the gills were assessed.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) fry (1.2 g; SD: 0.5) originating from Bornholms Lakseklækkeri (Denmark) were used in the present study. The fish were reared under disease-free conditions and were confirmed to be free of *F. psychrophilum* upon arrival and treated as previously described (Henriksen *et al.* 2013). In brief, the fish were acclimatized for 3 weeks and fed commercial feed daily (INICIO Plus, BioMar, Denmark). Fish weighing below 0.6 g and above 1.5 g were removed to reduce variation, before experimental groups were chosen randomly. The tanks used before and after exposure to H_2O_2 and immersion in *F. psychrophilum* were opaque to reduce unintentional stress. The present work was carried out in agreement with the internationally accepted guidelines for use of laboratory animals in research and under license by The Animal Experiments Inspectorate, Ministry of Justice, Copenhagen, Denmark (J.nr. 2006/561-1204).

2.2. Bacterial strain

A *F. psychrophilum* strain obtained from a Danish outbreak of RTFS in 1995 (950106-1/1) was used for challenge. The stock was stored at -80 °C in 15-20 % glycerol. Cultivation was carried out as previously described (Henriksen, Madsen *et al.* 2013). In brief, the bacterium was pre-cultivated in 10 mL tryptone yeast extract salts (TYES) broth at 15°C for 3 days (110 rpm). Then, 0.5 mL of the pre-culture was used to inoculate 100 mL TYES broth, which was further cultivated for 48 hours. The culture was inspected under microscope to verify purity and CFU were determined (10^{-4} to 10^{-7} in duplicates on TYES agar).

2.3. Challenge

Fish were challenged as previously described method (Henriksen, Madsen *et al.* 2013). In brief, the setup consisted of four groups of 45 fish in duplicate (n = 360) for sampling: A) Control, B) H_2O_2, C) *F. psychrophilum* and D) H_2O_2 + *F. psychrophilum*. The control group was immersed in 1:9

sterile TYB, the H_2O_2 group was immersed for 60 min in aerated 2 L tanks containing 150 mg H_2O_2 L^{-1}, the *F. psychrophilum* group was immersed in 1:9 broth culture diluted in water and containing 10^7 CFU mL^{-1} for 30 min and exposure to H_2O_2 preceded immersion in *F. psychrophilum* in the H_2O_2 + *F. psychrophilum*. Water temperature after challenge was 16.8 ± 0.5 °C.

2.4. Sampling

The head of 2 fish from each replicate (4 per group in total) were fixed in 10 % neutral buffered formalin for 24 h (room temperature) and subsequently stored in 70 % ethanol. The second gill arch was removed from the head, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). For qPCR, five fish were sampled from each replicate (10 per group in total) at each time point: 0 h, 4 h, 48 h, 125 h and 192 h post-challenge and fish were euthanized using MS-222. Gills were removed aseptically and placed in RNA*later* (Sigma Aldrich) and pre-stored for 48 hours at 4 °C and subsequently stored at -20 °C.

Mortality in the various groups was monitored in separate tanks (Henriksen, Madsen *et al.* 2013), and spleen, kidney and brain of fish dying in these tanks during the experimental period were sampled. Brain and kidney samples were streaked on TYES and blood agar plates (Dalsgaard & Madsen 2000), while the spleen was incubated in TYES broth at 110 rpm and streaked on TYES agar upon the presence of visible growth. The plates were incubated for 7 d at 15 °C for TYES and 20 °C for blood. The identity of re-isolated bacteria was confirmed using PCR (Wiklund, Madsen, Bruun & Dalsgaard 2000).

2.5. Microscopy

Microscopy was carried out using a Zeiss Axioimager M1 epifluorescence microscope equipped with a 120-W HBO lamp, and micrographs were taken with an AxioCam MRm v. 3 FireWiremonocrome camera using AxioVision software, v. 4.5 (Carl Zeiss).

2.6. RNA isolation and cDNA synthesis

RNA isolation and cDNA synthesis was carried as previously described (Henriksen, Kania, Buchmann & Dalsgaard 2013). A second arch from each fish was homogenized by using 5 mm stainless steel beads (Qiagen) with a TissueLyzer II (Qiagen). Total RNA was isolated from the samples with Maxwell® 16 LEV simplyRNA Tissue Kit, which integrated DNase treatment of the samples. The quality of RNA was determined spectrophotometrically by measuring the optical

density (OD) at 260/280 nm (NanoDrop 2000c, Thermo Scientific). Finally, the concentration in each sample was adjusted to the same level in all samples using nuclease free water. All steps involving RNA were carried out in a RNase-free environment and filtered tips and gloves were used, and RNA was stored at -80 °C.

cDNA was synthesized using TaqMan® Reverse Transcription and random Oligo(dT) primers (TaqMan® Reverse Transcription, Applied Biosystems). Reactions contained 400 ng RNA template and 2.0 µl 10x RT-buffer, 4.4 µl 25 mM $MgCl_2$, 4.0 µl 50 µM dNTP, 1.0 µl RNase Inhibitor and 1.0 µl Reverse Transcriptase. Reactions were adjusted to a total volume of 20 µl using RNase free water. Amplifications were run on a Biometra T- 3000 thermocycler amplifier (Biometra): 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. Nuclease free water was used to dilute cDNA 10 times before storage at −20 °C.

2.7. Real-time PCR: TaqMan® and SYBR-green

TaqMan® primers and probes used are listed in Table 1 (Henriksen *et al.* 2013). Samples originating from the same group were always run on the same plate (96-well microplates, AH Diagnostics as) and a single master mix was used for each assay. Each reaction volume was 12.5 µl: 0.5 µl of each primer (10 µM), 0.5 µl probe (5 µM) and 6.25µl JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, Denmark) and 2.25 µl nuclease free H_2O. ELF 1-α was used as a reference gene (Raida & Buchmann 2008; Ingerslev, Pettersen, Jakobsen, Petersen & Wergeland 2006; Olsvik, Lie, Jordal, Nilsen & Hordvik 2005) and negative controls from cDNA synthesis were also run. A Stratagene MX3005PTM real-time thermocycler (AH Diagnostics as) was used for all assays: 95°C for 15 min and 40 cycles of 95 °C for 30 s and 60 °C for 30 s.

A SYBR-green assay was used for quantification of *F. psychrophilum* (Orieux, Bourdineaud, Douet, Daniel & Le Hénaff 2011). Reactions consisted of 12.5 µl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 2 µmol L^{-1} of each primer. Nuclease free water was added to a total reaction volume of 25 µl. The following program was used: 96 °C for 10 min and 40 x 96 °C for 30 s, 56 °C for 30 s and 72 °C 30s. Cut-off was set at 31 cycles, corresponding to approximately 10 CFU according to the standard curve. At this point, all positive controls were detected and no unspecific amplification was observed in the uninfected controls. Efficiency for amplification was 100 % ± 5 % for all assays. A melting-curve analysis was performed to confirm absence of primer-dimer formation as well as confirm assay specificity.

2.8. Data analysis

For qPCR, the relative expression was determined using the $-^{\Delta\Delta}$Ct method (Livak & Schmittgen 2001) and expression of the reference gene ELF-1α was used for normalization. The normalized data was compared at each time-point using Student's T-test (1-tail) and changes were considered to be significant when both $p \leq 0.05$ and fold changes ≥ 3. Relative pathogen loads were also compared using Student's T-test and changes in expression of immune relevant genes were correlated with pathogen load using 1-way ANOVA and Tukey's post-test. For reisolation of *F. psychrophilum*, Fisher's exact test was used to see, whether exposure to H_2O_2 altered the distribution of *F. psychrophilum* in the examined tissues. Gene expression levels were correlated with the relative pathogen load measured as 16S rRNA in the individual groups using Pearson product-moment correlation coefficient. Pathogen load is stated as a relative value, as RNA samples did not originate from identical amounts of tissue.

3. Results

3.1. Gill morphology

The gills in the control groups showed normal morphology (Fig. 1A). Treatment with H_2O_2 disrupted the structure of secondary lamella and basis at the primary lamellae. The epithelial lifting and formation of edemas was most severe after 4 h (Fig. 1B). The nucleus of many cells was condensed and compressed, indicating necrosis. After 192 h, the gill structure approached the normal state again and the epithelial lifting was mostly gone (Fig. 1C). However, hyperplasia was detected on the secondary lamellae and some clubbing on secondary lamellae tips was also observed. Immersion in *F. psychrophilum* resulted in slight epithelial lifting at the basis of the secondary lamellae 4 h post-immersion (Fig. 1D). After 48 h and 125 h, more severe epithelial lifting was observed as well as the occasional formation of edemas and dilation of the brachial artery (Fig. 1E). The secondary lamellae also displayed clubbing at the tips. After 192 h, the overall gill structure approached normal morphology again, although surfaces of the secondary lamellae were still rugged and smaller edemas were visible (Fig. 1F). When combining H_2O_2 and *F. psychrophilum*, epithelial lifting was observed on the secondary lamella after 4 h (Fig. 1G). After 48 h and 125 h, a varying degree of epithelial lifting and condensed nuclei were observed and the branchial artery was dilated; the gill tissue in some samples was severely damaged. After 192 h, a greater extent of edemas and epithelial lifting was observed (Fig. 1H).

3.2. Relative pathogen load of *F. psychrophilum*

The relative pathogen load for each sampling is shown in Figure 2. A high level of *F. psychrophilum* 16S rRNA was observed 4 h post-exposure followed by a continued decline to a lower level. Treatment with H_2O_2 did not result in significant differences in the relative load of *F. psychrophilum* in the gills during the first 192 h after infection. However, a tendency towards a higher amount of *F. psychrophilum*-specific 16S rRNA was seen in the H_2O_2 + *F. psychrophilum group*.

3.3. Reisolation of *F. psychrophilum* from dead fish

It was possible to re-isolate the bacteria from spleen, kidney and brain following exposure to *F. psychrophilum*. Exposure to H_2O_2 before immersion in *F. psychrophilum* significantly decreased the occurrence of culturable *F. psychrophilum* in the spleen, while this was not the case for the brain and kidney (Table 2). However, the pattern of pathogen re-isolation in the kidneys from the two infected groups was similar to the distribution in the spleen with the lowest number of successful re-isolations in the H_2O_2 + *F. psychrophilum* group.

3.4. qPCR gene expression analysis

The constitutive expression of each gene is provided in Table 3; the variation is relatively low for most genes. Changes in gene expression are given in fold and are summarized in Table 4, while correlation between pathogen load and changes in gene expression are stated in Table 5. Regulation of IL-6 varied significantly between the replicates at several sampling points and was omitted from the data set.

After 4 h, a 3-5 fold up-regulation of IL-1β was observed for all groups, while a 3-fold up-regulation was seen for IL-17c1 in the H_2O_2+ *F. psychrophilum* group and an 8-fold down-regulation of IgT was seen in the *F. psychrophilum* group. No correlations between pathogen load and regulation of gene expression were observed.

After 48 h, a 4-fold down-regulation of IL-10 was seen for the H_2O_2+ *F. psychrophilum* group, while IL-17c1 was down-regulated 8-fold in both the H_2O_2 and H_2O_2+ *F. psychrophilum* groups. A positive correlation was found between pathogen load and regulation of IL-4/13A in the H_2O_2 + *F. psychrophilum* group.

After 125 h, IL-1β was up-regulated 9-fold in the *F. psychrophilum* group and SAA was up-regulated 4-fold in the H_2O_2+ *F. psychrophilum* group. In the group exposed to H_2O_2, an 8-fold down-regulation of IL-17c1, a 13-fold down-regulation of IgT and a 5-fold up-regulation of CD8 were observed. No correlations between pathogen load and regulation of gene expression were observed.

After 192 h, SAA was up-regulated 3-fold in the H_2O_2+ *F. psychrophilum* group and CD8 was up-regulated 4-fold in the *F. psychrophilum* group. In the *F. psychrophilum* group, negative correlations were observed between pathogen load and regulation of IL-17c1, MHC I and MHC II, while IgM positively correlated in the H_2O_2+ *F. psychrophilum* group.

4. Discussion

Previous investigations regarding the immune response to *F. psychrophilum* have primarily used injection-challenged or naturally infected fish (Evenhuis & Cleveland 2012; Orieux *et al.* 2013; Overturf & LaPatra 2006; Villarroel *et al.* 2008). Injection is a suboptimal approach in investigations regarding the immune response, since the mucosal surfaces are bypassed. The experimental model used in the present work has previously been used in a study on immune response in the head kidney (Henriksen *et al.* 2013) and included two infected groups: One group was immersed directly in diluted bacterial broth, while the other was exposed to H_2O_2 before immersion. Pre-treatment with H_2O_2 increased mortality of the infection two-fold: From 9.1 % to 19.2 % (Henriksen, Madsen *et al.* 2013).

Several studies have investigated the pathological effects of H_2O_2 on salmonids, in order to establish recommendations for safe application on fish (Tort *et al.* 2002; Arndt & Wagner 1997; Gaikowski, Rach & Ramsay 1999; Rach, Schreier, Howe & Redman 1997; Speare & Arsenault 1997), while fewer have investigated the histological changes. The gills of rainbow trout have been demonstrated to be the only tissue damaged by H_2O_2 (Tort, Jennings-Bashore, Wilson, Wooster & Bowser 2002). Observed gill lesions in rainbow trout and Atlantic salmon, *Salmo salar* L., included hyperplasia, hypertrophy, fused lamellae, clubbing at the tips of secondary lamellae, necrosis and lifting of the epithelium, wrinkled and folded epithelial layers, bleeding, necrosis and desquamation (Tort *et al.* 2002; Kiemer & Black 1997). The previously reported histological changes due to H_2O_2-exposure corresponded well with the damage seen in the present study, although fused lamellae were rare. Already after 192 hours, gill structure appeared mostly normal. Although mechanisms for tissue repair are poorly understood, the gills can recover from minor damage

relatively fast, while repair after comprehensive damage reportedly begins within weeks of exposure of H_2O_2 (Speare & Ferguson 2006). Immersion of rainbow trout in *F. psychrophilum* alone primarily resulted in epithelial lifting and formation of edemas. When combining pre-treatment with H_2O_2 with immersion in *F. psychrophilum*, epithelial lifting and edemas were still present after 192 hours.

Morphological changes were observed in the gills following immersion in a highly virulent *F. psychrophilum* strain and the presence of bacteria was confirmed using qPCR, although no bacteria could be seen in the tissue sections stained with H&E. If the bacterial cells were situated on the mucus, they may have been lost in the fixation or handling. If the bacterium did come into contact with the gill tissue, an immune reaction and the presence of inflammatory cells would be expected, unless the immune response is suppressed by the pathogen. Exposure to H_2O_2 before immersion in *F. psychrophilum* did not significantly alter the amount of *F. psychrophilum* 16S rRNA found in the gills in any of the samplings. After 4 hours, there was a relatively high amount of bacteria in both infected groups, but the level declined after 48 hours. Since no bacteria were observed in the gill sections, the bacteria may be situated on the outside of the gill tissue. Alternatively, the bacteria could have penetrated the gills during the first hours following infection and no longer be present in the gills. The pathogen may have gained easy access to the bloodstream through the gills, since the bacteria were isolated from the internal organs after experimental challenge. A study using scanning electron microscopy demonstrated that *F. psychrophilum* did not affect the gill tissue of immersion-challenged ayu, *Plecoglossus altivelis*. The presence of *F. psychrophilum* clusters between the gill lamellae were concluded to be accumulations rather than reflecting colonization, since the bacterium was only sporadically detected one hour post-infection (Kondo, Kawai, Kurohara & Oshima 2002). Highly virulent *F. psychrophilum* strains have been shown to adhere to rainbow trout gills in a perfusion model, resulting in edemas, epithelial lifting, sloughing of epithelial cells and disintegration of lamellar tissue, and *F. psychrophilum* clusters were observed at the tip of primary lamellae (Nematollahi, Decostere, Pasmans, Ducatelle *et al.* 2003). Another study of *F. psychrophilum* in ayu included naturally infected fish, injection-challenged fish and fish scarified with needles before immersion-challenge. *F. psychrophilum* was detected in several tissues in both groups using FISH, including the gills (Liu, Izumi & Wakabayashi 2001). Using the same technique and FISH protocol, preliminary results suggest that *F. psychrophilum* was not present in the gills of rainbow trout fry after immersion in *F. psychrophilum* (author's unpublished results).

Flavobacterium psychrophilum could be reisolated from a significantly higher number of spleens in the *F. psychrophilum* group compared to the H_2O_2 + *F. psychrophilum* group. A similar pattern was seen in the kidney, although the results were not statistically significant. The spleen is a key source of antibody production and immunological memory in rainbow trout (Speare *et al.* 2006) containing monocytes, macrophages and dendritic cells (Lovy, Wright & Speare 2008). Thus, the increased amount of bacteria in the spleen in the non-H_2O_2 exposed group may reflect a mounting immune response, although splenic macrophages have previously been shown to be tolerant to *F. psychrophilum* cells, thus potentially providing a place of refuge for the pathogen (Nematollahi, Decostere, Pasmans & Haesebrouck 2003).

The IL-1β gene was up-regulated immediately after exposure to H_2O_2, to *F. psychrophilum* and to both combined. Up-regulation of SAA and down-regulation of IL-10 was only demonstrated in the H_2O_2 + *F. psychrophilum* group, which also had the most damaged gills. Thus, indications of a pro-inflammatory response were observed in both infected groups, although the response manifested itself slightly differently, which was also the case in the head kidney (Henriksen *et al.* 2013) . No correlation with pathogen load was found for SAA, IL-1β or IL-10. SAA is an acute phase protein, which recruits leukocytes early during an infection (Kania & Chettri 2013), and IL-10 is an anti-inflammatory cytokine controlling expression of e.g. IL-6 and IL-1β. A positive correlation between regulation of IL-10 and *F. psychrophilum* pathogen-load was demonstrated in the head kidney of fish pre-treated with H_2O_2 (Henriksen *et al.* 2013), suggesting an immune-suppressive effect on the host. Although IL-10 has an immune suppressive function, it is believed to have multiple effects resulting in an antibody-based T_h2-type response (Raida *et al.* 2008; Zou, Clark & Secombes 2003; Hummelshoj, Ryder & Poulsen 2006). SAA was also found to be up-regulated in the gills of rainbow trout which were naturally infected with *F. psychrophilum* (Villarroel *et al.* 2008).

IL-17c1 is one of two homologue IL-17 genes in rainbow trout, and it has been shown to be expressed mainly in skin and gills (Wang, Martin & Secombes 2010). Initially, IL-17c1 was up-regulated in the H_2O_2 + *F. psychrophilum* group and followingly down-regulated. Furthermore, a negative correlation of IL-17c1 was found in the *F. psychrophilum* group after 192 hours. The effect of IL-17c1 and IL-17c2 (Wang *et al.* 2010) in fish is still unknown. In mammals, IL-17 recruits monocytes and neutrophiles to the site of infection. In the head kidney, a positive correlation with pathogen load was initially observed in the *F. psychrophilum*-exposed group after 125 hours, while a negative correlation was observed after 192 hours (Henriksen *et al.* 2013).

IgT was down-regulated in the *F. psychrophilum* group immediately after exposure to the pathogen, and a positive correlation between pathogen load and regulation of IgM was found in the H_2O_2 + *F. psychrophilum* group at the last sampling. In a previous study of the head kidney (Henriksen *et al.* 2013), IgM regulation also correlated with pathogen load eight days post-infection in the infected group pre-treated with H_2O_2. IgM is the predominant antibody in teleosts and is found in the blood, while IgT is a mucosal antibody. IgT has been found to have a high constitutive expression in the gills of rainbow trout larvae (Chettri, Raida, Kania & Buchmann 2012), although that was not the case in the fry used in present study.

In the present study, no changes in regulation for CD4, MHC I, MHC II and IL-4/13A were observed in the infected groups, while CD8 was significantly up-regulated at the latest time-point in the *F. psychrophilum* group. A positive correlation with pathogen load was seen for IL-4/13A in the H_2O_2 + *F. psychrophilum* group, while MHC I and MHC II negatively correlated in the *F. psychrophilum* group after 192 hours. IL-4/13A decreases the production of T_h1 cells, macrophages and dendritic cells, while inducing naive T cells to differentiate into T_h2 cells, which are related to antibody production. CD8 positive cells recognize antigens presented by the MHC I molecule and are linked to intracellular infections and cytotoxic cells. CD4 positive cells, on the other hand, recognize antigens presented on MHC II molecules and are linked to extracellular infections and antibody production. The presence of CD8, MHC II, IgT and IgM positive cells has been demonstrated in gill tissue in adult rainbow trout, which would allow for a fast immune response upon contact with invading pathogens (Olsen *et al.* 2011). Expression of CD8 was demonstrated to be repressed in the spleen of fish naturally infected with *F. psychrophilum* (Orieux *et al.* 2013), while injection-challenge led to an increased expression in the spleen (Overturf & LaPatra 2006) of rainbow trout. In the head kidney, no regulation was found after exposure to *F. psychrophilum*, although a positive correlation between regulation of CD4 and MHC I and pathogen load was observed (Henriksen *et al.* 2013).

Exposure to H_2O_2 resulted in an up-regulation of IL-1β and CD8 and a down-regulation of IL-17c1 and IgT in the gills. When comparing changes in regulation between the three treated groups, exposure to H_2O_2 before immersion in the bacteria was found to have several effects on the immune response: IL-17c1 was up-regulated immediately after infection and no down-regulation of IgT was seen. Later, an up-regulation of IL-1β was absent in the H_2O_2 + *F. psychrophilum* group, while SAA was only up-regulated in this group. At the latest sampling, SAA was also found to be up-regulated in this group only, while CD8 was only up-regulated in the *F. psychrophilum* group. IL-

4/13A and IgM were only positively correlated with pathogen load in the H_2O_2 + *F. psychrophilum* group, while IL-17c1, MHC I and MHC II only correlated negatively in the *F. psychrophilum* group. Consequently, exposure to H_2O_2 does not only affect the immune response to *F. psychrophilum* in the head kidney (Henriksen *et al.* 2013), but also in the gills.

The immune response in the gills of rainbow trout fry to experimental infection with *F. psychrophilum* was sparse, but exposure to H_2O_2 before infection altered the response. H_2O_2 had a severe impact on gill morphology, and infection with *F. psychrophilum* intensified the damages and delayed healing. Although the presence of *F. psychrophilum* 16S rRNA was demonstrated using qPCR, no bacteria were observed in the H&E stained tissue sections. At the time of death, the pathogen was found in several organs, demonstrating a systemic spread of infection. The gills as a potential portal of entry in RTFS are a possibility, although bacterial adherence could not be proven in the present work.

Acknowledgements

This work was performed under the Danish Fish Immunology Research Centre and Network (DAFINET) and was supported by the Danish Council of Strategic Research. The technical assistance provided by Lene Gertman during sampling is greatly appreciated.

References

Arndt R.E. & Wagner, E.J. (1997) The toxicity of hydrogen peroxide to rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clarki* fry and fingerlings. *Journal of the World Aquaculture Society* **28,** 150-157.

Avendaño-Herrera R., Magariños, B., Irgang, R. & Toranzo, A.E. (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). *Aquaculture* **257,** 104-110.

Chettri J.K., Raida, M.K., Kania, P.W. & Buchmann, K. (2012) Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*. *Developmental and comparative immunology* **36,** 463-474.

Dalsgaard I. & Madsen, L. (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *Journal of Fish Diseases* **23,** 199-209.

Declercq A.M., Van den Broeck, W., Haesebrouck, F., Bossier, P., Chiers, K., Dewulf, J., Cornelissen, M. & Decostere, A. (2013) Interactions of highly and low virulent *Flavobacterium columnare* strains with the gills of carp (*Cyprinus carpio*). *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book,* Tampere, Finland, pp. 126.

Deshmukh S., Kania, P.W., Chettri, J.K., Skov, J., Bojesen, A.M., Dalsgaard, I. & Buchmann, K. (2013) Insight from Molecular, Pathological, and Immunohistochemical Studies on Cellular and Humoral Mechanisms Responsible for Vaccine-Induced Protection of Rainbow Trout against *Yersinia ruckeri*. *Clinical and vaccine immunology* **20,** 1623-1641.

Evenhuis J.P. & Cleveland, B.M. (2012) Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. *Veterinary immunology and immunopathology* **146,** 8-17.

Gaikowski M.P., Rach, J.J. & Ramsay, R.T. (1999) Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. *Aquaculture* **178,** 191-207.

Henriksen M.M.H., Madsen, L. & Dalsgaard, I. (2013) Effect of hydrogen peroxide on immersion challenge of rainbow trout fry with *Flavobacterium psychrophilum*. *PLoS ONE* **8(4)**.

Henriksen M.M.H., Kania, P.W., Buchmann, K. & Dalsgaard, I. (2013) Evaluation of the immune response in rainbow trout fry, *Oncorhynchus mykiss* (Walbaum), after exposure to *Flavobacterium psychrophilum* and/or hydrogen peroxide. *Journal of Fish Diseases,* in press.

Holt R.A., Rohovec J.S. & Fryer J.L. (1993) Bacterial coldwater disease. In: *Bacterial Diseases of Fish (ed. by V. Inglis, R.J. Roberts & N.R. Brombage),* pp. 3-23. Blackwell Scientific Publications, Oxford.

Hummelshoj L., Ryder, L.P. & Poulsen, L.K. (2006) The role of the interleukin-10 subfamily members in immunoglobulin production by human B cells. *Scandinavian Journal of Immunology* **64,** 40-47.

Ingerslev H.C., Pettersen, E.F., Jakobsen, R.A., Petersen, C.B. & Wergeland, H.I. (2006) Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). *Molecular immunology* **43,** 1194-1201.

Jayasuriya N.S., Herath, T.K., Bron, J.E., Adams, A., Mullins, J., McGurk, C. & Thompson, K.D. (2013) Gene expression profiles in Atlantic salmon gills after vaccination and challenge with *Aeromonas salmonicida* sub species *Salmonicida*. *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 94.

Kania P.W. & Chettri, J.K. (2013) Serum Amyloid A Gene expression and immunohistochemical localization in rainbow trout, *Oncorhynchus mykiss*, infected by *Yersinia ruckeri*. In: *DAFINET Workshop and Ph.D. Course: Book of abstracts*, Copenhagen, Denmark, pp. 27.

Kato G., Takano, T., Sakai, T., Matsuyama, T. & Nakayasu, C. (2013) *Vibrio Anguillarum* bacterin introduced through gill epithelium induces inflammatory responses in gills of Japanese flounder *Paralichthys Olivaceus*. *In: 16th EAFP International Conference on Diseases of Fish and Shellfish: Abstract book*, Tampere, Finland, pp. 95.

Kiemer M.C.B. & Black, K.D. (1997) The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. *Aquaculture* **153,** 181-189.

Khimmakthong U., Deshmukh, S., Chettri, J.K., Bojesen, A.M., Kania, P.W., Dalsgaard, I. & Buchmann, K. (2013) Tissue specific uptake of inactivated and live *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*): Visualization by immunohistochemistry and in situ hybridization. *Microbial pathogenesis* **59-60,** 33-41.

Kondo M., Kawai, K., Kurohara, K. & Oshima, S. (2002) Adherence of *Flavobacterium psychrophilum* on the body surface of the ayu *Plecoglossus altivelis*. *Microbes and Infection* **4,** 279-283.

Liu H., Izumi, S. & Wakabayashi, H. (2001) Detection of *Flavobacterium psychrophilum* in various organs of ayu *Plecoglossus altivelis* by in situ hybridization. *Fish Pathology* **36,** 7-11.

Livak K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* **25,** 402-408.

Lorenzen E. (1994) Studies on *Flexibacter psychrophilus* in relation to rainbow trout fry syndrome (RTFS). PhD thesis, Royal Veterinary and Agricultural University, Copenhagen

Lorenzen E., Dalsgaard, I., From, J., Hansen, E.M., Hørlyck, V., Korsholm, H., Mellergaard, S. & Olsen, N.J. (1991) Preliminary investigations of fry mortality syndrome in rainbow trout. *Bulletin of the European Association of Fish Pathologists* **11,** 77-79.

Lovy J., Wright, G.M. & Speare, D.J. (2008) Comparative Cellular Morphology Suggesting the Existence of Resident Dendritic Cells Within Immune Organs of Salmonids. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* **291,** 456-462.

Madetoja J., Dalsgaard, I. & Wiklund, T. (2002) Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. *Diseases of aquatic organisms* **52,** 109-118.

Nematollahi A., Decostere, A., Pasmans, F., Ducatelle, R. & Haesebrouck, F. (2003) Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss*. *Diseases of aquatic organisms* **55,** 101-107.

Nematollahi A., Decostere, A., Pasmans, F. & Haesebrouck, F. (2003) *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases* **26,** 563-574.

Olsen M.M., Kania, P.W., Heinecke, R.D., Skjoedt, K., Rasmussen, K.J. & Buchmann, K. (2011) Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: Molecular and immunohistochemical studies. *Fish & shellfish immunology* **30,** 859-869.

Olsvik P.A., Lie, K.K., Jordal, A.E., Nilsen, T.O. & Hordvik, I. (2005) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC molecular biology* **6,** 21.

Orieux N., Bourdineaud, J., Douet, D., Daniel, P. & Le Hénaff, M. (2011) Quantification of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), tissues by qPCR. *Journal of Fish Diseases* **34,** 811-821.

Overturf K. & LaPatra, S. (2006) Quantitative expression (Walbaum) of immunological factors in rainbow trout, *Oncorhynchus mykiss* (Walbaum), after infection with either *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, or infectious haematopoietic necrosis virus. *Journal of Fish Diseases* **29,** 215-224.

Rach J.J., Schreier, T.M., Howe, G.E. & Redman, S.D. (1997) Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *Progressive Fish-Culturist* **59,** 41-46.

Raida M.K. & Buchmann, K. (2008) Temperature-dependent immune response in rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri* O1.

Raida M.K. & Buchmann, K. (2008) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri* . *Fish & shellfish immunology* **25,** 533-541.

Speare D.J. & Arsenault, G.J. (1997) Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 2653-2658.

Speare D.J. & Ferguson H.W. (2006) Gills and pseudobranches. In: *Systemic pathology of fish*, 2nd edition (ed. by H.W. Ferguson), pp. 25-63. Scotian Press, London, UK.

Thomas-Jinu S. & Goodwin, A.E. (2004) Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. *Journal of Fish Diseases* **27,** 23-28.

Tobback E., Decostere, A., Hermans, K., Ryckaert, J., Duchateau, L., Haesebrouck, F. & Chiers, K. (2009) Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout *Oncorhynchus mykiss* . *Diseases of aquatic organisms* **84,** 219-228.

Tort M.J., Jennings-Bashore, C., Wilson, D., Wooster, G.A. & Bowser, P.R. (2002) Assessing the effects of increasing hydrogen peroxide dosage on rainbow trout gills utilizing a digitized scoring methodology. *Journal of Aquatic Animal Health* **14,** 95-103.

Villarroel F., Zambrano, A., Amthauer, R. & Concha, M.I. (2009) Detection of up-regulated serum amyloid A transcript and of amyloid AA aggregates in skeletal muscle lesions of rainbow trout infected with *Flavobacterium psychrophilum*. *Veterinary immunology and immunopathology* **130,** 120-124.

Villarroel F., Casado, A., Vásquez, J., Matamala, E., Araneda, B., Amthauer, R., Enriquez, R. & Concha, M.I. (2008) Serum amyloid A: A typical acute-phase reactant in rainbow trout? *Developmental and comparative immunology* **32,** 1160-1169.

Wang T., Martin, S.A.M. & Secombes, C.J. (2010) Two interleukin-17C-like genes exist in rainbow trout *Oncorhynchus mykiss* that are differentially expressed and modulated. *Developmental & Comparative Immunology* **34,** 491-500.

Wiklund T., Madsen, L., Bruun, M.S. & Dalsgaard, I. (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. *Journal of applied microbiology* **88,** 299-30

Zou J., Clark, M.S. & Secombes, C.J. (2003) Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, *Fugu rubripes*. *Immunogenetics* **55,** 325-335.

Gene	Fwd	Rev	Probe	Bp	Acc. no.
EF-1α	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	63	AF498320
FoxP3a	CTACAGGCACAGCCTGTCACTAGG	GCTCCTCTGGCTCTTTAGTGG	CCAGAACCGAGGTGGAGTGTCACG	80	FM883710
FoxP3b	TCCTGCCCCAGTACTCATCCC	GCTCCTCTGGCTCTTTAGTGG	CTTGGCAGCAGATGGAGTGCCACG	75	FM883711
IL-17c1	CTGGCGGTACAGCATCGATA	GAGTTATATCCATAATCTTCGTATTCGGC	CGTGATGTCCGTGCCCTTTGACGATG	138	FM955453
IL-17c2	CTGGCGGTACAGCATCGATA	CAGAGTTATATGCATGATGTTGGGC	CGTGGTGTCCAGGCCCTTTAATGATG	134	FM955454
IL-10	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTTC	CATCGGAAACATCTTCCACGAGCT	70	AB118099
IL-6	ACTCCCCTCTGTCACACACC	GGCAGACAGGTCCTCCACTA	CCACTGTGCTGATAGGGCTGG	91	DQ866150
IgT	AGCACCAGGGTGAAACCA	GCGGTGGGTTCAGAGTCA	AGCAAGACGACCTCCAAAACAGAAC	73	AY870265
IgM	CTTGGCTTGTTGACGATGAG	GGCTAGTGGTGTTGAATTGG	TGGAGAGAACGAGCAGTTCAGCA	72	S63348
SAA	GGGAGATGATTCAGGGTTCCA	TTACGTCCCCAGTGGTTAGC	TCGAGGACACGAGGACTCAGCA	79	AM422446
CD4	CATTAGCCTGGGTGGTCAAT	CCCTTTCTTTGACAGGGAGA	CAGAAGAGAGAGCTGGATGTCTCCG	89	AY973028
CD8	ACACCAATGACCACAACCATAGAG	GGGTCCACCTTTCCCACTTT	ACCAGCTCTACAACTGCCAAGTCGTGC	74	AF178054
MHC I	TCCCTCCCTCAGTGTCT	GGGTAGAAACCTGTAGCGTG	CAGAAGACCCCCTCCTCTCCAGT	73	AY523661
MHC II	TGCCATGCTGATGTGCAG	GTCCCTCAGCCAGGTCACT	CGCCTATGACTTCTACCCCAAACAAAT	68	AF115533
TcR-β	TCACCAGCAGACTGAGAGTCC	AAGCTGACAATGCAGGTGAATC	CCAATGAATGGCACAAACCAGAGAA	73	AF329700
IL-4/13A	ATCCTTCTCCTCTCTGTTGC	GAGTGTGTGTGTATTGTCCTG	CGCACCGGCAGCATAGAAGT	139	AB574337
IL-1β	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG	CATGGAGAGGTTAAAGGGTGGC	91	AJ223954

Table 1: Probes and primers (Henriksen *et al.* 2013).

Spleen*			
	Fish #	+	−
F.p.	30	**27 (90 %)**	3 (10 %)
H_2O_2 + *F.p.*	69	**51 (74 %)**	18 (26 %)
Fish #	99	78	21

Kidney			
	Fish #	+	−
F.p.	28	24 (86 %)	4 (14 %)
H_2O_2 + *F.p.*	64	47 (73 %)	17 (27 %)
Fish #	92	71	21

Brain			
	Fish #	+	−
F.p.	31	23 (74 %)	8 (26 %)
H_2O_2 + *F.p.*	72	52 (72 %)	20 (28 %)
Fish #	103	75	28

Table 2: Reisolation of *F. psychrophilum* from spleen. kidney and brain of rainbow trout fry after immersion in either *F. psychrophilum* (*F.p.*) or H_2O_2 and *F. psychrophilum* (H_2O_2 + *F.p.*). The pathogen was either successfully reisolated (+) or not found (-). The number of fish is stated along with the percentage in parenthesis. Statistical significance is indicated by an asterisk * (= $p < 0.05$).

Gene	C_t	SEM
EF1-α	22.35	0.22
IL-10	30.75	0.23
IL-6	38.20	0.37
IL-1β	31.18	0.30
SAA	23.23	0.23
IL-17c1	31.52	0.34
IL-17c2	35.47	0.52
FoxP3a	31.74	0.50
FoxP3b	31.09	0.23
IgT	33.10	0.79
IgM	27.23	0.15
CD8	28.31	0.62
CD4	33.78	0.37
MHC I	25.50	0.31
MHC II	22.26	0.35
IL-4/13A	31.22	0.23
TcR-β	26.64	0.24

Table 3: Constitutive gene expression for genes examined by qPCR

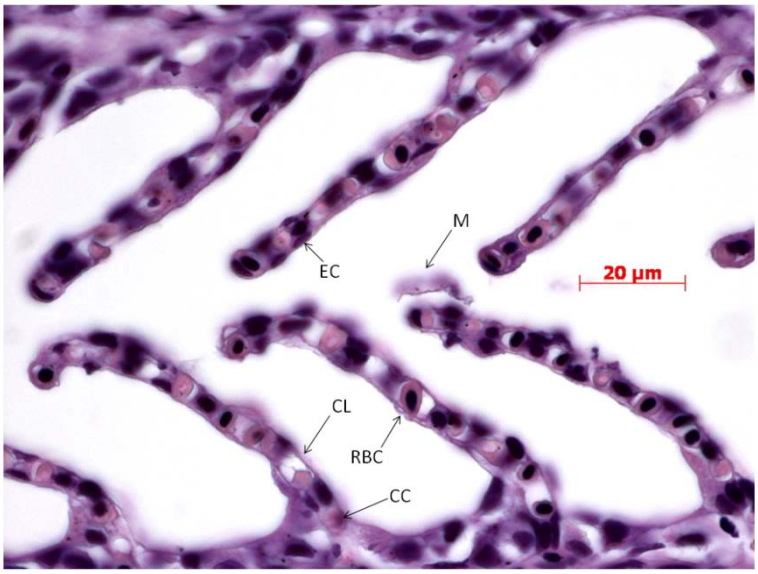
	4 h						48 h					
	H_2O_2	SEM	*F.p.*	SEM	H_2O_2 + *F.p.*	SEM	H_2O_2	SEM	*F.p.*	SEM	H_2O_2 + *F.p.*	SEM
IL-10	1.27	0.27	-1.02	0.49	1.38	0.22	-2.30	0.38	-2.68	0.41	**-3.73** **	0.43
IL-1β	**4.73** **	0.71	**3.54** **	0.60	**2.99** **	0.54	-1.08	0.44	-1.07	0.56	-1.83	0.26
SAA	-1.33	0.27	-1.68	0.19	-1.01	0.27	-1.41	0.11	-1.45	0.26	-2.15	0.26
IL-17c1	-1.46	0.76	2.09	0.59	**3.47** **	0.41	**-7.79** ***	0.61	-2.49	0.85	**-7.94** ***	0.55
IL-17c2	1.68	0.57	1.95	0.80	1.07	0.50	-2.76	0.40	-2.55	0.57	-2.96	0.61
FoxP3a	1.58	0.29	1.20	0.35	1.79	0.30	-1.65	0.21	-1.14	0.41	-1.59	0.26
FoxP3b	-1.07	0.30	-1.20	0.42	1.83	0.22	-1.60	0.30	-1.58	0.44	-1.64	0.22
IgT	2.60	0.30	**-7.89** *	1.11	-1.71	0.75	-1.37	0.37	-2.85	1.09	-1.74	0.36
IgM	-1.50	0.36	-1.57	0.27	-1.44	0.28	-1.96	0.23	-1.78	0.32	-1.79	0.24
CD8	3.10	0.69	1.13	0.71	3.71	0.89	1.11	0.67	8.85	2.80	6.14	3.00
CD4	1.43	0.61	-1.29	0.52	1.99	0.48	-1.84	0.47	-1.07	0.45	7.31	3.66
MHC I	1.17	0.28	1.48	0.32	1.64	0.29	-1.79	0.40	-1.28	0.32	-1.76	0.30
MHC II	-1.28	0.41	1.37	0.40	1.43	0.26	-1.15	0.30	1.10	0.31	1.17	0.41
IL-4/13A	-1.48	0.58	1.29	0.26	1.64	0.27	-1.34	0.29	-1.28	0.27	-1.40	0.33
TcR-β	1.66	0.35	1.62	0.49	2.16	0.48	-1.17	0.48	1.72	0.53	-1.31	0.38
	125 h						**192 h**					
	H_2O_2	SEM	*F.p.*	SEM	H_2O_2 + *F.p.*	SEM	H_2O_2	SEM	*F.p.*	SEM	H_2O_2 + *F.p.*	SEM
IL-10	1.15	0.44	3.54	0.95	1.22	1.02	-2.60	0.29	1.01	0.87	1.45	0.84
IL-1β	1.03	0.56	**9.06** *	1.28	2.38	1.13	-2.43	0.84	1.03	0.87	1.07	0.66
SAA	1.28	0.59	4.19	1.16	**4.46** *	1.04	1.41	0.37	2.13	0.39	**3.08** *	0.64
IL-17c1	**-8.41** ***	0.31	2.16	1.35	-3.12	1.22	-2.41	0.48	2.55	1.02	-2.03	0.74
IL-17c2	1.49	0.47	3.97	1.23	1.35	1.12	-3.75	0.86	-1.13	1.67	1.06	1.15
FoxP3a	1.20	0.61	3.57	1.48	2.56	0.79	-1.40	0.46	1.55	0.98	2.67	1.03
FoxP3b	1.10	0.34	2.55	1.28	1.56	0.93	-1.13	0.31	1.72	0.88	1.90	0.75
IgT	**-13.35** ***	0.94	1.07	0.48	2.03	1.30	-2.21	0.36	1.37	1.43	1.13	0.96
IgM	-10.87	0.69	-16.69	0.20	-8.57	1.01	-1.16	0.45	1.35	0.48	2.48	1.01
CD8	**5.15** *	0.95	3.61	1.25	1.35	0.75	2.14	0.55	**3.64** *	0.66	2.47	1.08
CD4	2.73	0.48	5.20	1.35	2.85	0.80	1.98	0.49	3.91	1.11	2.99	0.86
MHC I	-1.82	0.44	1.82	1.34	1.97	1.05	-1.25	0.40	-1.05	0.49	1.04	0.87
MHC II	-1.27	0.37	1.80	1.34	1.41	1.02	1.25	0.45	1.42	0.33	1.83	0.68
IL-4/13A	1.51	0.44	3.83	1.49	3.02	1.11	-1.49	0.21	1.18	0.86	1.52	0.79
TcR-β	2.04	1.81	2.45	1.20	3.11	1.14	-2.83	0.47	-1.91	0.54	-1.26	0.49

Table 4: Gene expression in fold (min 3-fold change) and SEM 4. 48. 125 and 192 hours after immersion in H_2O_2. *F. psychrophilum* (*F.p.*) or both. Significance levels are indicated by asterisks; asterisks (* = $p < 0.05$. ** = $p < 0.01$ and *** = $p < 0.001$).

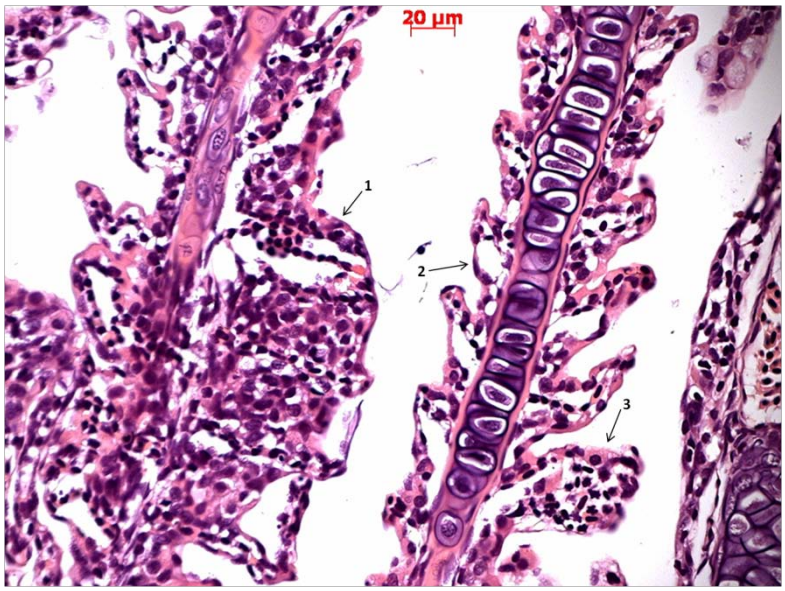
Time	4t		48t		125t		192t	
	-H_2O_2	**+H_2O_2**	**-H_2O_2**	**+H_2O_2**	**-H_2O_2**	**+H_2O_2**	**-H_2O_2**	**+H_2O_2**
FoxP3 b								
FoxP3 a								
IL-17 c1							**-0.85***	
IL-17 c2								
IL-10								
IgT								
IgM								**0.81****
SAA								
CD8								
CD4								
MHC I							**-0.82***	
MHC II							**-0.92****	
TcR-β								
IL-4/13				**0.79***				
IL-1β								

Table 5: Correlation between gene expression and relative pathogen load for the two infected groups; either without (-) or with (+) H_2O_2 pre-treatment. Negative correlations are denoted by "-" and significance levels are indicated by asterisks (* = $p < 0.05$ and ** = $p < 0.01$).

Figure 1. H&E-stained gill samples. Control group (A): 1:9 TYB for 30 min; H_2O_2 group (B-C): 150 mg L^{-1} for 60 min; *F. psychrophilum* group (D-G): 1:9 bacterial broth with 10^7 CFU mL^{-1} for 30 min; H_2O_2 + *F. psychrophilum* group (H-K): 150 mg L^{-1} for 60 min followed by immersion in a 1:9 bacterial broth with 10^7 CFU mL^{-1} for 30 min.

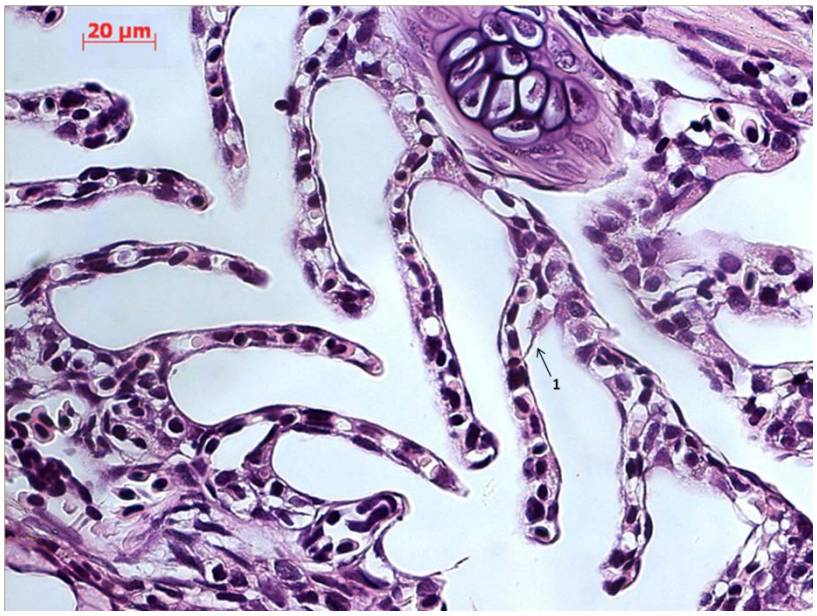


A) Control (4 h). No morphological changes were observed at any time-point. EC: epithelial cell, M: mucus, CL: capillary lumen, RBC: red blood cell, CC: chloride cell.

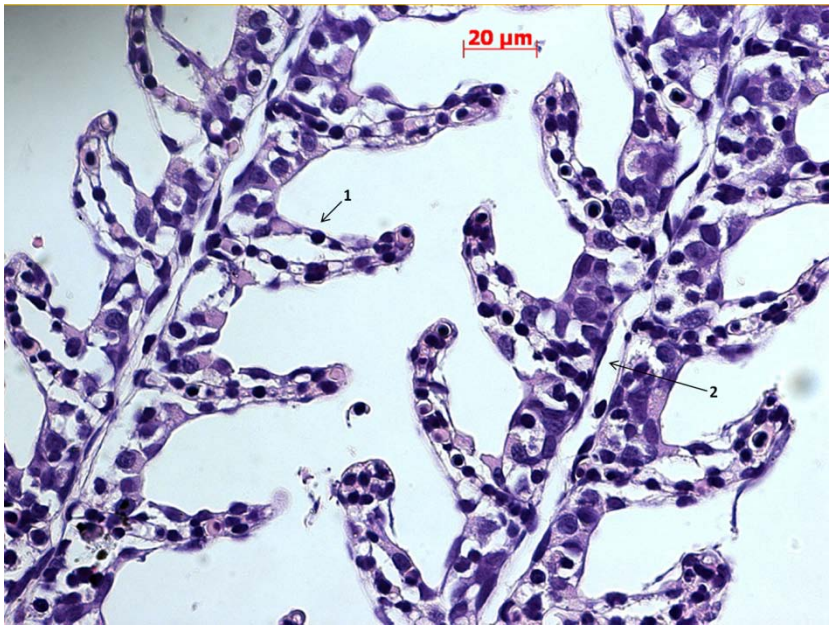


B) H_2O_2 (4 h). Lamellar fusion (1) and epithelial lifting with edema formation (2) of the secondary arches. Condensed nuclei suggest a necrosis of the cells (3).

C) H_2O_2 (192 h). Epithelial lifting at the basis of the secondary lamellae (1). The gill structure approached the normal state again and epithelial lifting was mostly gone. Hyperplasia is seen on the secondary lamellae and clubbing of the tips (2).



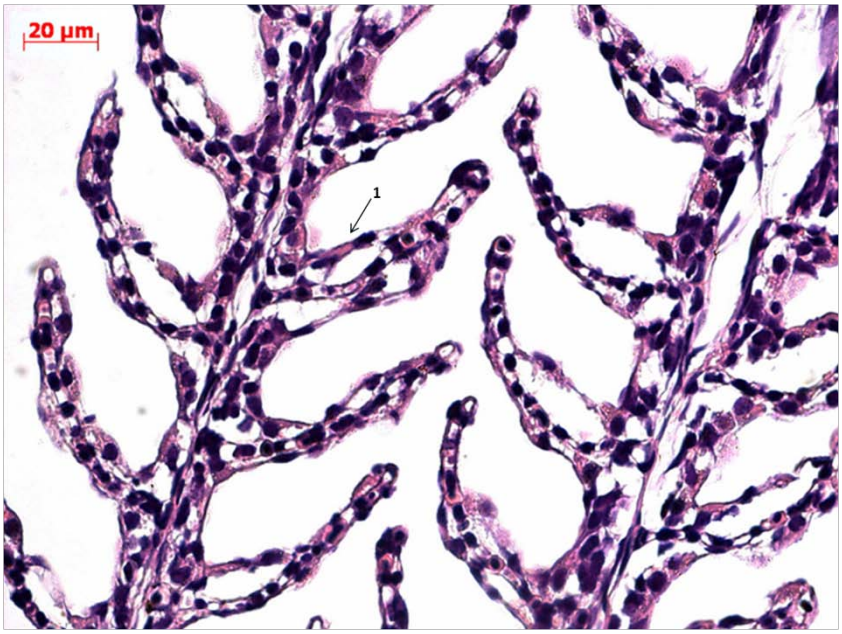
D) *F. psychrophilum* (4 h). Epithelial lifting was observed at the basis of the secondary lamellae (1).



E) *F. psychrophilum* (48 h). Epithelial lifting was intensified and formation of edemas was observed (1). The brachial artery was dilated (2) and the primary lamellae were thickened.

F) *F. psychrophilum* (192 h). The general structure appeared more normal, although secondary filaments were irregular and some epithelial lifting (1) was still visible.

G) H_2O_2 + *F. psychrophilum* (4 h). Epithelial lifting and formation of edemas (1) on the secondary filaments.



H) H_2O_2 + *F. psychrophilum* (192 h). The gill filaments were severely damaged with edemas and epithelial lifting (1).

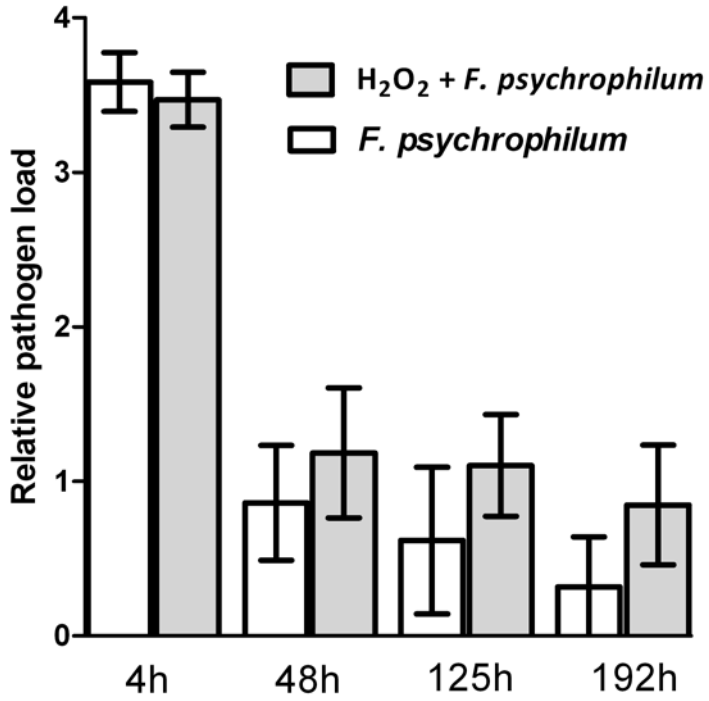


Figure 2: Relative pathogen load assed by qPCR and shown as log(C_t). The white bars denote the *F. psychrophilum* infected group, while the grey bars denotes the H_2O_2 pre-treated and *F. psychrophilum* infected group.